PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Michael G. Rosenblum	Group Art Unit: 1642
Serial No.: 10/676,725	Examiner: Goddard, Laura B.
Filed: October 1, 2003	Atty. Dkt. No.: CLFR:029USD1
For: NOVEL ANTIBODY DELIVERY SYSTEM FOR BIOLOGICAL RESPONSE MODIFIERS	

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September 11, 2008
Date

David L. Parker

APPEAL BRIEF

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PATENT

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BRIEF ON APPEAL

Commissioner for Patents
P. O. Box 1450
Alexandria, VA 22313-1450

Commissioner:

Appellants hereby submit this Appeal Brief to the Board of Patent Appeals and Interferences pursuant to 37 C.F.R. §41.31(a)(1) and 41.37 in light of the Final Office Action dated April 28, 2008 (the "Action") and Notice of Appeal filed July 25, 2008.

The fee for filing this Appeal Brief is $255.00. The required fee is being charged to a credit card through EFS-Web concurrently with this submission. The Commissioner is hereby authorized to deduct any underpayment of fees or any additional fees required under 37 C.F.R. §§ 1.16 to 1.21 in connection with the filing of this paper from Fulbright & Jaworski L.L.P. Account No.: 50-1212/CLFR:029USD1.

I. REAL PARTY IN INTEREST

The real party in interest is the assignee, Research Development Foundation.

II. RELATED APPEALS AND INTERFERENCES

There are no interferences or appeals for related cases.

III. STATUS OF THE CLAIMS

Claims 1-6, 8-9, 11-12, 20 and 22 are cancelled. Claims 15 and 17-19 are withdrawn. Claims 7, 10, 13-14, 16, 21, and 23 – 32 are currently rejected and are the subject this appeal.

IV. STATUS OF AMENDMENTS

There are no un-entered amendments.

V. SUMMARY OF THE CLAIMED SUBJECT MATTER

The following summary of the claimed subject matter is made with reference to the Specification, and claims as filed or if appropriate after entry of any amendments. The references to the Specification are provided as examples, and should not be considered an exhaustive list of support for each claim. Support can also be found in the claims as-filed.

Independent Claim 26 (see claims appendix) is directed to a method of treating cancer in a human patient in need of such treatment (claims 5, 9, and 10 as-filed; Specification p.6 ll. 14- 19; p.9 ll. 17-22) wherein the method comprises steps of (a) identifying a patient having a tumor, which tumor comprises cells for targeting and wherein those cells comprise a cell surface antigenic marker at concentrations in excess of that found at other non-target sites (Specification p.13 line 1- p.15 line 8); (b) obtaining a composition comprising a protein with an antigen

recognition site directed toward a cell surface associated antigen conjugated or fused to the biological response modifier (*Id.* p.4 ll. 10-21, pp. 9-10), wherein it has been determined that cells of the patient's cancer express an antigen recognized and bound by the protein with an antigen recognition site (*Id.* p. 5 line 13- p.6 line 3; p.12 ll. 3-13); and (c) administering an amount of the composition to the patient effective to treat the cancer (*Id.* p.9 ll. 18-23).

Dependent Claim 23 (see claims appendix) is directed to the method of claim 26 (see above) wherein the protein with an antigen recognition site is fused to a biological response modifier (*Id.* p.9 ll. 6-14).

Dependent Claim 16 (see claims appendix) is directed to the method of claim 23 (see above), wherein the biological response modifier is the cytokine TNF-alpha. (*Id.* p.10 ll. 13-26).

Dependent Claim 31 (see claims appendix) is directed to the method of claim 26 (see above), wherein the cancer is cervical cancer. (*Id.* p.9 ll. 20-22).

VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

A. Are claims 7, 24, 26-29 and 30 improperly rejected as obvious under 35 U.S.C § 103(a) over U.S. Patent No. 4,753,894 to Frankel *et al.* ("Frankel") in view of U.S. Patent No. 4,771,128 to Ferris ("Ferris") ?

B. Are claims 7, 10, 13, 14, 21, 24-29 and 32 improperly rejected as obvious under 35 U.S.C § 103(a) over U.S. Patent No. 4,590,071 to Scannon *et al.* ("Scannon") in view of U.S. Patent No. 4,771,128 to Ferris ("Ferris") as evidenced by Kirkwood *et al.*, J Clin. Oncology 5:1247-1255 (1987) ("Kirkwood") ?

C. Is claim 16 improperly rejected as obvious under 35 U.S.C § 103(a) over U.S. Patent No. 4,590,071 to Scannon *et al.* ("Scannon") in view of U.S. Patent No. 4,771,128 to Ferris ("Ferris") in further view of Blick *et al.*, Cancer Res. 47:2986-2989 (1987) ("Blick") ?

D. Is claim 23 improperly rejected as obvious under 35 U.S.C § 103(a) over U.S. Patent No. 4,590,071 to Scannon *et al.* ("Scannon") in view of U.S. Patent No. 4,771,128 to Ferris ("Ferris") in further view of Ghose *et al.*, Crit Rev. Ther. Drug Carrier Sys. 3:263-359 (1987) ("Ghose") ?

E. Are claims 7, 24, 26-29 and 31 improperly rejected as obvious under 35 U.S.C § 103(a) over U.S. Patent No. 4,666,845 to Mattes *et al.* ("Mattes") in view of U.S. Patent No. 4,771,128 to Ferris ("Ferris") ?

VII. ARGUMENT

A. Claims 7, 24, 26-29 and 30 are improperly rejected as obvious under 35 U.S.C § 103(a) over U.S. Patent No. 4,753,894 to Frankel et al. ("Frankel") in view of U.S. Patent No. 4,771,128 to Ferris ("Ferris")

The Examiner rejects claims 7, 24, 26-29 and 30 as obvious over the combination of Frankel *et al.* (US 4,753,894; Exhibit 1; "Frankel") in view of Ferris (US 4,771,128; Exhibit 2; "Ferris"). However, the instant claims require (1) determining that the patient's tumor comprises cells that (2) express a cell surface antigenic marker that (3) binds the protein with an antigen recognition site at (4) concentrations in excess of that found at other non-target sites, as shown below.

> Claim 26: A method of treating cancer in a human patient in need of such treatment, the method comprising the steps of:
>
> (a) ***identifying a patient having a tumor***, which tumor comprises cells for targeting and wherein those cells comprise a cell surface antigenic marker at concentrations in excess of that found at other non-target sites;
>
> (b) ***obtaining a composition comprising a protein with an antigen recognition site*** directed toward a cell surface associated antigen conjugated or fused to the biological response modifier, ***wherein it has been determined that cells of the patient's cancer express an antigen recognized and bound by the protein with an antigen recognition site;*** and
>
> (c) administering an amount of the composition to the patient effective to treat the cancer.

Claim 26, emphasis added. These elements ***are not accounted for in Frankel or Ferris*** and, thus, a *prima facie* case of obviousness has not been presented. M.P.E.P. § 2143.03.

The Examiner argues that the patient in Frankel, "treated with an antibody conjugate specific for breast cancer would ***necessarily*** have been identified or diagnosed as a patient having a breast tumor and the patient's breast cancer ***would be expressing*** the breast cancer-specific antigen targeted by the antibody conjugate for killing tumor cells," and "Applicants are arguing the ***inherent nature*** of the patient's cancer." Action, pp.18, 20 emphasis added. Yet this is absolutely untrue. ***None*** of the antibodies presented in Frankel bind to ***all*** of the breast cancer cells lines or tissue sections tested in excess of that found at other non-cancerous sites. Frankel, Tables I, II, and III. Therefore, there are breast cancer cell lines, tissues, and ***tumors*** that ***do not express*** antigens to Frankel's antibodies. At most, a given Frankel patient's cancer might express a given antigen, but the fact that a certain result or characteristic ***may*** occur or be present in the prior art is not sufficient to establish the inherency of that result or characteristic. *In re Rijckaert*, 9 F.3d 1531, 1534, 28 USPQ2d 1955, 1957 (Fed. Cir. 1993).

The Examiner further alleges in the Action that "[g]iven the breast cancer patients being treated with the immunoconjugates are identified as having breast cancer, the cells of the breast tumor in the patient would express an antigen recognized and bound by the antibodies in the immunoconjugates taught by Frankel." Action, p.20, ¶2. This is again incorrect! Take, for example, the breast cancer tissue sections of the breast cancer labeled "R" in Table 2 of Frankel. The only three antibodies that bind to R also bind to a significant amount of normal tissue types. Frankel, Table 1, 2G3, 245E7, and 280D11. Here is a case where breast cancer has been diagnosed, but the breast cancer ***does not express an antigen recognized by Frankel's antibodies*** at higher concentrations than normal tissue. Thus, it is not true as the Examiner alleges, that just because a patient has been "identified as having breast cancer," that the patient will express an antigen recognized by the antibodies taught by Frankel. Further, it is well known

in the art of cancer therapy that individualized treatment, *i.e.*, treating individual subjects for antigen expression, is a recent goal and it would not have been considered to be obvious at the time.

The Examiner appears to rely on "common knowledge" to show inherent properties that Appellants show do not exist, thus Appellants requested that the Examiner support such a finding with adequate evidence. Office Action Response, dated January 11, 2008, p.9, ll.3-5. The Examiner has an affirmative duty to provide such requested evidence. M.P.E.P. § 2144.03C. But the Examiner utterly failed to appropriately respond in the Final Office Action, likely because the Examiner ***cannot provide such evidence*** and Frankel itself refutes the Examiner's argument.

The Examiner also fails to take into account the teachings of Frankel as a whole. M.P.E.P. § 2141.02 VI. Frankel teaches the importance of the "range of human breast cancer cells to which [antibodies] bind..." Frankel col. 3, ll.18-23. Frankel teaches treating ***any patient with breast cancer, regardless of whether or not they express the specific antigen***. If Frankel was directed to individualized treatment and physical detection of the antigens to which the antibodies bind, Frankel would not emphasize the range of breast cancer cells to which Frankel's antibodies bind. Thus, a limitation of the instant claims, that a determination be made that the patient's cancer expresses the ***targeted antigen***, has not been accounted for by Frankel and would not have been obvious to one of skill in the art given the disclosure of Frankel.

The Examiner admits that "Frankel does not teach that the antibody is conjugated to a biological response modifier." Action, p. 18, ¶ 4. The Examiner instead relies on Ferris for this teaching. However, Ferris does not address the deficiencies of the Examiner's rejection

described above. Namely, Ferris does not account for determining if a patient's tumor express a certain antigen.

In view of all of the above, appellants respectfully request the Examiner's rejections be reversed.

B. Claims 7, 10, 13, 14, 21, 24-29 and 32 are improperly rejected as obvious under 35 U.S.C § 103(a) over U.S. Patent No. 4,590,071 to Scannon et al. ("Scannon") in view of U.S. Patent No. 4,771,128 to Ferris ("Ferris") as evidenced by Kirkwood et al., J. Clin. Oncology 5:1247-1255 (1987) ("Kirkwood")

The Examiner rejects claims 7, 10, 13, 14, 21, 24-29 and 32 as obvious over the combination of Scannon *et al.* (US 4,590,071; Exhibit 3; "Scannon") in view of Ferris, as evidenced by Kirkwood *et al.* J. CLin. Oncology 5: 1247 – 1255, 1987 (Exhibit 4; "Kirkwood"). Appellants respectfully traverse.

A person of skill in the art at the time the application was filed would not be motivated to combine the references with a reasonable expectation of success and, thus, the Examiner fails to make a *prima facie* case of obviousness. M.PE.P. §§ 2143.01-02. Specifically, the Examiner's rejection of claims 7, 10, 13, 14, 21, 24-29 and 32 is predicated upon an assumption that Scannon teaches a 240kD melanoma antigen that is a ***cell surface*** antigenic marker at a concentration in excess of that found in non-target sites. This assumption has no basis, as no where does Scannon discuss the location of the antigenic marker disclosed therein. Appellants show that it was known in the art that 1) an intracellular antigen can serve as a target for immunotoxin therapy (which rebuts any presumption that such a target is necessarily cell surface), and 2) at least some 240K tumor antigens are intracellular in nature. U.S. Patent No. 4,894,227, col. 5 ll.34-36; col. 10 ll. 14-16 (Exhibit 5; "Stevens"). Thus, a person of skill in the art at the time the invention was made ***would not*** assume Scannon teaches antigens on the cell surface.

In an attempt to provide an accounting for the antigen location that Scannon lacks, the Examiner relies upon Kirkwood, which discusses an antibody that recognizes a melanoma antigen identified as "gp240" that is said to be a cell surface glycoprotein antigen. Action, p.4, ¶2. However, the single cited page of Kirkwood does not necessarily teach the same "240kD" antigen of Scannon. Action, p.4, ¶2 citing Kirkwood p. 1247. Scannon also does not teach a "240kD" antigen that is also a glycoprotein. In fact, the Examiner conceded that, "the claimed antigen ***appears*** to be the same as the prior art...." Action, p.4, ¶2, emphasis added. But the Examiner offers no foundation for this assessment. Nonetheless, the Examiner argues that the burden is shifted to Appellants to show otherwise. *Id.* at pp.4-5. This is not true.

The fact that a certain characteristic ***may*** be present in the prior art is not sufficient to establish the inherency of that result or characteristic. *In re Rijckaert*, 9 F.3d 1531, 1534, 28 USPQ2d 1955, 1957 (Fed. Cir. 1993). The burden shifts only if the claimed and prior art products are identical or substantially identical, or are produced by identical or substantially identical processes. *In re Best*, 562 F.2d 1252, 1255 (CCPA 1977). That is not the case here, where the Examiner offers only guesses as to whether the claimed antigenic marker is the same as that in certain prior art references, and Appellants have shown a person of skill in the art would not immediately equate them.

The Examiner also cites four references to demonstrate that, "the antibody conjugates taught by Scannon inherently bind the cell surface melanoma antigen recognized by antibody ZME-018." Action, p.6, ¶3 through p.7, ¶1; Exhibits 6-9. Yet, this approach is unavailing to the Examiner because it requires reliance upon a reference from 2006! Action, p.7, ¶2; Exhibit 6. Although inherency does not necessarily require recognition of a characteristic in the prior art, without such recognition, the combination of references in this case cannot be said to show the

required combination of known elements by known processes with no more than what would be expected at the time. M.P.E.P. § 2143.02. Without the 2006 reference, the Examiner makes no argument that a person of skill would believe the teachings of Scannon ***could be*** combined with the teaching of Kirkwood and Ferris and ***would be*** expected to result in, "a protein with an antigen recognition site directed toward ***a cell surface associated antigen*** conjugated or fused to the biological response modifier." Claim 26, emphasis added. "Obviousness cannot be predicated on what is not known at the time an invention is made, even if the inherency of a certain feature is later established." M.P.E.P. § 2141.02 citing *In re Rijckaert*, 9 F.2d 1531, 28 USPQ2d 1955 (Fed. Cir. 1993).

Further, the Examiner has failed to account for a limitation of the instant claims - the determination that cells of the patient's cancer express an antigen recognized and bound by the protein with an antigen recognition site. See Independent claim 26. To establish a *prima facie* case of obviousness all claim limitations must be accounted for. MPEP § 2145. At the time the instant Application was filed, the focus of cancer treatment was on treating all subjects as if the all were afflicted with the same or similar "generic" type of cancer. This is demonstrated by the prevalence of cocktail treatment regimes in the prior art that combine multiple types of drugs into one treatment plan. Ghose, *et al.*, Crit Rev. Ther. Drug Carrier Sys. 3:263-359 (1987) ("Ghose") at p. 335, ¶2. The instant claims, however, are directed to treating a specific subpopulation of cancer patients that express a specific antigen. This limitation is not met by the Examiner's citations.

In view of the above, the Examiner's burden has not been carried and Appellants respectfully request the Examiner be reversed.

C. Claim 16 is improperly rejected as obvious under 35 U.S.C § 103(a) over U.S. Patent No. 4,590,071 to Scannon et al. ("Scannon") in view of U.S. Patent No. 4,771,128 to Ferris ("Ferris") in further view of Blick et al., Cancer Res. 47:2986-2989 (1987) ("Blick")

The Examiner rejects claim 16 as obvious over the combination of Scannon in view of Ferris, in further view of Blick *et al.*, Cancer Res. 47:2986-2989, 1987 (Exhibit 10; "Blick"). Appellants respectfully traverse.

Again, the Examiner fails to make a *prima facie* case that Claim 16 is obvious in view of the cited references. First, Claim 16 is not obvious for all the reasons described in "B" above, because the claim from which it depends is not obvious. M.P.E.P. §2143.03; *In re Fine*, 837 F.2d 1071, 5 USPQ2d 1596 (Fed. Cir. 1988). Second, the Examiner attempts to show obviousness of dependent Claim 16 without the Kirkwood reference (a reference that the Examiner apparently felt was required to reject independent Claim 26). But the Examiner then fails to show the now-cited Blick reference teaches (1) that the "240kD" antigen of Scannon and the antigen in the claims are the same antigen, and (2) that the additional limitation that TNF is TNF-alpha as required in Claim 16.

Specifically, Blick merely relates to a phase I study using unconjugated TNF alpha that was administered by i.v. or i.m. injection. The Examiner argues that Blick teaches TNF alpha has anti-tumor effects. Action p.11, ¶6. Yet Blick does not relate to immunotoxins or similar targeted therapy at all. Thus, a person of skill in the art reading Blick would have very little reason to believe that whatever "success" was reported therein could be repeated by a targeted TNF alpha conjugate, particularly considering that no evidence of record is presented that such a conjugate would retain TNF activity. A person of skill in the art at the time the application was

filed would, thus, not be motivated to combine the references with a reasonable expectation of success. M.PE.P. §§ 2143.01-02.

Further, the Examiner has failed to account for the limitation of the instant claims that requires the determination that cells of the patient's cancer express an antigen recognized and bound by the protein with an antigen recognition site. Independent claim 26. This limitation has been described in detail in sections "A" and "B" above and has again not been accounted for by the Examiner.

The Examiner has failed to carry the burden of making a *prima facie* case for obviousness. Appellants therefore respectfully request the Board reverse the Examiner's rejection.

D. Claim 23 is improperly rejected as obvious under 35 U.S.C § 103(a) over U.S. Patent No. 4,590,071 to Scannon *et al.* ("Scannon") in view of U.S. Patent No. 4,771,128 to Ferris ("Ferris") in further view of Ghose.

The Examiner rejects claim 23 as obvious over the combination of Scannon in view of Ferris, in further view of Ghose *et al.*, Crit Rev. Ther. Drug Carrier Sys. 3:263-369, 1987 (Exhibit 11; "Ghose"). Appellants respectfully traverse.

The Examiner also fails to make a *prima facie* case that Claim 23 is obvious in view of the cited references. As with Claim 16 discussed above, Claim 23 is not obvious because the claim from which it depends is not obvious. M.P.E.P. §2143.03; *In re Fine*, 837 F.2d 1071, 5 USPQ2d 1596 (Fed. Cir. 1988). Additionally, the Examiner attempts to show obviousness of dependent Claim 23 without the Kirkwood reference (a reference which the Examiner apparently felt was required to reject independent Claim 26), yet fails to show the now-cited Ghose reference teaches both what Kirkwood was cited for as well as the additional limitations in Claim 23.

As explained in section B above, a *prima facie* case of obviousness has not been presented by the Examiner with the combination of Scannon and Ferris. The addition of Ghose to teach only that some antibodies may be fused to some biological response modifiers does not address the deficiencies in the Examiner's rejections. Further, none of the references cited here account for the determination that the patients cancer express a certain antigen as described in section "A" and "B" above.

Thus, Appellants respectfully request the Examiner's rejections be reversed.

E. Claims 7, 24, 26-29 and 31 are improperly rejected as obvious under 35 U.S.C § 103(a) over U.S. Patent No. 4,666,845 to Mattes *et al.* ("Mattes") in view of U.S. Patent No. 4,771,128 to Ferris ("Ferris")

Lastly, the Action rejects claims 7, 24, 26-29 and 31 as obvious over the combination of Mattes *et al.* (US 4,666,845; Exhibit 12; "Mattes") in view of Ferris. Appellants respectfully traverse.

All of the claims are allowable over the above cited art, however, Appellants note that Claim 31 expressly stands and falls alone. Claim 31 is specifically directed to targeting cervical cancer and no reference in the record has been cited that correctly accounts for such a limitation.

The Examiner alleges that Mattes teaches that the "monoclonal antibody MH49 binds to an antigen found on human cervical carcinoma cells at concentrations in excess of that found in other tissues (Table 1, Table II, col. 4, ll.15-15; col. 11, l.55 through col. 12, l.18; col. 13, ll.1-11)." Presumably, the Examiner is citing the antibody MH94, as this is the antibody referenced in the Examiners citations, and no reference to MH49 is found in Mattes. More importantly, MH94 ***is not expressed on human cervical carcinoma cells at concentration in excess of that found in other tissues*** as the Examiner alleges. The Examiner has misread and misinterpreted

the data presented in Mattes, and as such, the current rejection has no basis in fact. Evidence of these mistakes is found at the Examiner's cited columns and line numbers and detailed below.

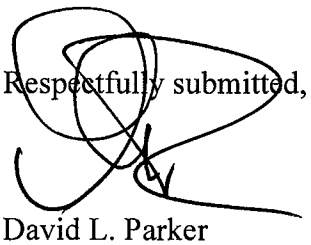
Table 1 in Mattes shows that mAb MH94 binds ***weakly*** to cervical carcinoma cells ME 180. Table II in Mattes shows the MH94 binds to 1/1 cervical carcinoma cells lines. Table II also describes that MH94 ***binds to normal*** pancreas, ureter, breast, prostate, cervix, urinary bladder epithelial, sweat and sebaceous gland cells. Further, Table II also demonstrates that MH94 ***binds to normal*** fetal stomach, intestine, pancreases, ureter, urinary bladder, uterus and cervix epithelial cells. Mattes also describes MH94 binding to normal cell types. Mattes, col. 11, l.55 through col. 12, l.18. The above shows an antigen (MH94) that binds only weakly to cervical carcinoma cell lines, but which also ***binds an excess of normal adult and fetal tissues***. Clearly, the limitation that the cells comprise a cell surface antigenic marker at concentrations in excess of that found at other non-target sites of the instant claims has not been accounted for in the Examiner's rejection.

The Examiners also argues that all cervical cancers will express the antigen to MH94. However, Mattes only tested one line. Further, Mattes does not disclose detecting each patient for the antigen prior to treatment. Thus, the limitation that each patient is tested for expression of the antigen has also not been accounted for, and the Examiner has not established a *prima facie* argument.

The rejection should be reversed as the Examiner has not met the burden of establishing a *prima facie* case of obvious and the rejection also lacks factual basis on this record.

VIII. CONCLUSION

In view of the above, Appellants believe that the foregoing remarks fully respond to all outstanding matters for this application. Appellants respectfully request that the Board reverse the rejections of all claims.



Respectfully submitted,

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Date: September 11, 2008

IX. CLAIMS APPENDIX

7. The method of claim 26, wherein said cancer is selected from the group consisting of breast cancer, cervical carcinoma and melanoma.

10. The method of claim 7, wherein the patient has been diagnosed with cancer and cells of the cancer express an antigen recognized by monoclonal antibody ZME-018 (ATCC accession number HB 11009), and further wherein the protein is a monoclonal antibody that recognizes and binds the antigen.

13. The method of claim 24, wherein the biological response modifier is a cytokine.

14. The method of claim 13, wherein the cytokine is TNF.

16. The method of claim 14, wherein the TNF is TNF-alpha.

21. The method of claim 24, wherein the protein's antigen recognition site recognizes and binds to the ZME-018 antigen, an antigen recognized by monoclonal antibody ZME-018 (ATCC accession number HB 11009).

23. The method of claim 26, wherein the protein with an antigen recognition site is fused to the biological response modifier.

24. The method of claim 26, wherein the protein with an antigen recognition site is conjugated to the biological response modifier.

25. The method of claim 14, wherein the protein's antigen recognition site recognizes and binds to the ZME-018 antigen, an antigen recognized by monoclonal antibody ZME-018 (ATCC accession number HB 11009).

26. A method of treating cancer in a human patient in need of such treatment, the method comprising the steps of:

(a) identifying a patient having a tumor, which tumor comprises cells for targeting and wherein those cells comprise a cell surface antigenic marker at concentrations in excess of that found at other non-target sites;

(b) obtaining a composition comprising a protein with an antigen recognition site directed toward a cell surface associated antigen conjugated or fused to the biological response modifier, wherein it has been determined that cells of the patient's cancer express an antigen recognized and bound by the protein with an antigen recognition site; and

(c) administering an amount of the composition to the patient effective to treat the cancer.

27. The method of claim 26, wherein the patient is diagnosed as having a tumor with a specific antigenic determinant that will allow targeting and concentration of the biological response modifier at the site where it is needed to kill tumor cells.

28. The method of claim 26, wherein the protein is an antibody.

29. The method of claim 28, wherein the antibody is a monoclonal antibody.

30. The method of claim 7, wherein the cancer is breast cancer.

31. The method of claim 7, wherein the cancer is cervical carcinoma.

32. The method of claim 7, wherein the cancer is melanoma.7.

X. EVIDENCE APPENDIX

Exhibit 1 – Frankel *et al.* (U.S. Patent No. 4,753,894) ("Frankel") first cited by the Appellants on 7/6/2004 in form PTO-1449 and made of record by the Examiner on 9/14/2005.

Exhibit 2 – Ferris *et al.*, (U.S. Patent No. 4,771,128) ("Ferris") first cited by the Appellants on 7/6/2004 in form PTO-1449 and made of record by the Examiner on 9/14/2005.

Exhibit 3 – Scannon *et al.* (U.S. Patent No. 4,590,071) ("Scannon") first cited by the Appellants in form PTO-1449 on 5/31/2005 and made of record by the Examiner on 9/14/2005.

Exhibit 4 – Kirkwood *et al.*, (J. Clin. Oncology, 5:1247-1255) ("Kirkwood") first cited by the Appellants in form PTO-1449 on 5/31/2005 and made of record by the Examiner on 9/14/2005.

Exhibit 5 – Stevens *et al.*, (U.S. Patent No. 4,894,227) ("Stevens") first cited by the Appellants in form PTO-1449 on 5/31/2005 and made of record by the Examiner on 9/14/2005.

Exhibit 6 – Ashcroft *et al.* (Chem Commun, 2006, 3004-3006) ("Ashcroft") first cited by the Examiner in form PTO-892 on 4/28/2008.

Exhibit 7 – Ferrone *et al.* (J of Dermatology, December 1988, 457-465) first cited by the Examiner in form PTO-892 on 4/28/2008.

Exhibit 8 – Martin *et al.* (Human gene Therapy, 1998, 9:737-746) first cited by the Examiner in form PTO-892 on 4/28/2008.

Exhibit 9 – Oratz *et al.* (J Biol Response Mod, 1990, 9:345-354) first cited by the Examiner in form PTO-892 on 4/28/2008.

Exhibit 10 - Blick *et al.,* (Cancer Research, 1987, 47:2986-2989) ("Blick") first cited by the Examiner in form PTO -892 on 9/14/2005.

Exhibit 11 - Ghose *et al.,* (Crit Rev Ther Drug Carrier Syst, 1987, 3:263-359) ("Ghose") first cited by the Examiner in form PTO -892 on 9/14/2005.

Exhibit 12 – Mattes *et al.* (U.S. Patent No. 4,666,845) ("Mattes") first cited by the Examiner in form PTO-892 on 9/11/2007.

Exhibit 1

United States Patent [19]

Frankel et al.

[11] **Patent Number: 4,753,894**

[45] **Date of Patent: Jun. 28, 1988**

[54] **MONOCLONAL ANTI-HUMAN BREAST CANCER ANTIBODIES**

[75] Inventors: **Arthur E. Frankel**, Palo Alto; **David B. Ring**, Redwood City; **Michael J. Bjorn**, Hercules, all of Calif.

[73] Assignee: **Cetus Corporation**, Emeryville, Calif.

[21] Appl. No.: **690,750**

[22] Filed: **Jan. 11, 1985**

Related U.S. Application Data

[63] Continuation-in-part of Ser. No. 577,976, Feb. 8, 1984, abandoned.

[51] **Int. Cl.**[4] **A61K 39/00**; G01N 33/54; C12N 5/00; C12R 1/91

[52] **U.S. Cl.** **436/548**; 435/240.27; 435/948; 530/387; 530/402; 530/808; 424/85; 935/104; 935/107

[58] **Field of Search** 435/68, 172.2, 240, 435/241, 948; 436/548; 424/85; 935/104, 107, 110

[56] **References Cited**

U.S. PATENT DOCUMENTS

4,172,124	10/1979	Koprowski et al.	435/240
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[57] **ABSTRACT**

Murine monoclonal antibodies are prepared and characterized which bind selectively to human breast cancer cells, are IgGs or IgMs, and when conjugated to ricin A chain, exhibit a TCID 50% against at least one of MCF-7, CAMA-1, SKBR-3, or BT-20 cells of less than about 10 nM. Methods for diagnosing, monitoring, and treating human breast cancer with the antibodies or immunotoxins made therefrom are described.

29 Claims, No Drawings

MONOCLONAL ANTI-HUMAN BREAST CANCER ANTIBODIES

CROSS-REFERENCE TO RELATED APPLICATION

This application is a continuation-in-part of copending U.S. application Ser. No. 577,976 filed Feb. 8, 1984.

DESCRIPTION

1. Technical Field

This invention is in the fields of immunology and cancer diagnosis and therapy. More particularly it concerns murine monoclonal anti-human breast cancer antibodies, hybridomas that produce those antibodies, immunochemicals made from those antibodies, and diagnostic and therapeutic methods that use those immunochemicals.

2. Background Art

Since the mid-1970s, there have been numerous reports of murine monoclonal antibodies that interact with human breast cancer associated antigens. In these reported studies, mice were immunized and boosted with human milk fat globule proteins, breast cancer cell lines or breast cancer membrane extracts. Immune splenocytes were fused with mouse myeloma cells and hybridomas were selected based on some specificity of the culture media for breast or breast cancer antigens. Taylor-Papadimitriou, J., et al, Int J Cancer (1981) 28: 17–21; Yuan, D., et al, JNCI (1982) 68: 719–728; Ciocca, D. R., et al, *Cancer Res* (1982) 42: 4256–4258. The normal tissue reactivities of these prior antibodies are different than the normal tissue reactivities of the antibodies of the present invention.

Numerous prior workers have suggested or reported linking cytotoxic agents to antibodies to make "immunotoxins." Recent interest has centered on immunotoxins of monoclonal antibodies conjugated to the enzymatically active portions (A chains) of toxins of bacterial or plant origin via heterobifunctional agents. Nevelle, D. M. and Youle, R. J., *Immunol Rev* (1982) 62: 75–91; Ross, W. C. J., et al, *European J Biochem* (1980) 104; Vitteta, E. S., et al, *Immunol Rev* (1982) 62: 158–183; Raso, V., et al, *Cancer Res* (1982) 42: 457–464; Trowbridge, I. W. and Domingo, D. L., *Nature* (*Cond*) (1981) 294: 171–173.

A principal aspect of the invention concerns murine monoclonal antibodies that:

(a) bind selectively to human breast cancer cells;

(b) are IgGs or IgMs;

(c) when conjugated to ricin A chain exhibit a tissue culture inhibitory dose which results in 50% of control (untreated) protein synthesis (TCID 50%) of less than about 10 nM against at least one of MCF-7, CAMA-1, SKBR-3, or BT-20 cells.

Preferred embodiments of these antibodies are those designated 260F9, 113F1, 2G3, 280D11, 266B2, 33F8, 245E7, 454C11, 317G5, 520C9, and 369F10, and functional equivalents thereof.

The murine x murine hybridomas that produce the above described antibodies are progeny of those hybridomas are other aspects of the invention.

Another aspect of the invention relates to immunotoxins that are conjugates of

(a) the above described monoclonal antibodies, and

(b) a cytotoxic moiety.

Another aspect of the invention concerns labeled derivatives of the above described monoclonal antibodies that are labeled with a detectable label that permits the derivatives to be used in diagnosing or monitoring human breast cancer.

Another aspect of the invention concerns a method of killing human breast cancer cells by contacting the cells with a cytocidally effective amount of one or more of the above described immunotoxins.

Other aspects of the invention are direct and indirect immunoassays for determining whether a human cell is a breast cancer cell. These assays involve incubating the cells with the monoclonal antibodies or labeled derivatives thereof. When the labeled derivatives are used, the presence of labeled binary immune complexes on the cells is read directly. When unlabeled antibody is used the cells are further incubated with a labeled antibody against the monoclonal antibody and the presence of labeled ternary immune complexes on the cells is read.

Modes for Carrying Out the Invention

As used herein the term "monoclonal antibody" means an antibody composition having a homogeneous antibody population. It is not intended to be limited as regards the source of the antibody or the manner in which it is made.

As used herein with respect to the exemplified murine monoclonal anti-human breast cancer antibodies, the term "functional equivalent" means a monoclonal antibody that: (a) crossblocks an exemplified monoclonal antibody; (b) binds selectively to human breast cancer cells; (c) has a G or M isotype; (d) binds to the same antigen as determined by immunoprecipitation or sandwich immunoassay; and (e) when conjugated to ricin A chain, exhibits a TCID 50% against at least one of MCF-7, CAMA-1, SKBR-3, or BT-20 cells of less than about 10 nM.

As used herein with regard to the monoclonal antibody-producing hybridomas of the invention the term "progeny" is intended to include all derivatives, issue, and offspring of the parent hybridoma that produce the monoclonal anti-human breast cancer antibody produced by the parent, regardless of generation or karyotypic identity.

Monoclonal Antibody Production

The antibody-producing fusion partners that are used to make the hybridomas of this invention are generated by immunizing mice with live human breast cancer cells or membrane extracts made therefrom. The mice are inoculated intraperitoneally with an immunogenic amount of the cells or extract and then boosted with similar amounts of the immunogen. Spleens are collected from the immunized mice a few days after the final boost and a cell suspension is prepared therefrom for use in the fusion.

Hybridomas are prepared from the splenocytes and a murine tumor partner using the general somatic cell hybridization technique of Kohler, B. and Milstein, C., *Nature* (1975) 256: 495–497 as modified by Buck, D. W., et al, *In Vitro* (1982) 18: 377–381. Available murine myeloma lines, such as those from the Salk Institute, Cell Distribution Center, San Diego, Calif., USA, may be used in the hybridization. Basically, the technique involves fusing the tumor cells and splenocytes using a fusogen such as polyethylene glycol. After the fusion the cells are separated from the fusion medium and grown in a selective growth medium, such as HAT medium, to eliminate unhybridized parent cells. The

hybridomas are expanded, if desired, and supernatants are assayed for anti-human breast cancer activity by conventional immunoassay procedures (e.g., radioimmunoassay, enzyme immunoassay, or fluorescence immunoassay) using the immunizing agent (breast cancer cells or membrane extract) as antigen. Positive clones are characterized further to determine whether they meet the criteria of the invention antibodies.

Hybridomas that produce such antibodies may be grown in vitro or in vivo using known procedures. The monoclonal antibodies may be isolated from the culture media or body fluids, as the case may be, by conventional immunoglobulin purification procedures such as ammonium sulfate precipitation, gel electrophoresis, dialysis, chromatography, and ultrafiltration, if desired.

Monoclonal Antibody Selection/Characterization

The important characteristics of the monoclonal antibodies are (1) their immunoglobulin class, (2) their selectivity for human breast cancer cells and the range of human breast cancer cells to which they bind and (3) their usefulness in making effective anti-human breast cancer immunotoxins.

The selectivity and range of a given antibody is determined by testing it against panels of (1) human breast cancer tissues and cells and (2) normal human tissues or cells of breast or other origin. In selecting the claimed antibodies approximately twenty-two thousand growing hybridoma cultures were initially screened against the immunizing breast tumor membranes or cell line, a panel of eight normal tissue membranes, a fibroblast cell line and a breast tumor frozen section. Clones that reacted with the neoplastic materials but not the normal materials were identified in this initial screen and chosen for isotyping and additional screening for selectivity and range. The additional screening involved: sixteen normal tissue sections, five normal blood cell types, eleven nonbreast neoplasm sections, twenty-one breast cancer sections and fourteen breast cancer cell lines. Antibodies were deemed to bind selectively to breast cancer if they bound strongly to less than about $\frac{1}{3}$ of the normal tissues and blood cell types. One hundred twenty-seven antibodies were purified and tested on the additional screen.

Antibodies exhibiting acceptable selectivity and range were conjugated to ricin A chain using N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP) or iminothiolane (IT) as a coupling agent. The conjugates were tested against MCF-7, CAMA-1, SKBR-3, and BT-20 cells in a 24-hour tissue culture assay. Sixteen of the antibodies exhibited acceptable immunotoxin activity (TCID 50% of less than 10 nM) against at least one of these breast tumor lines. Seven of the sixteen were found to recognize the same 210,000 dalton antigen, with six of the seven probably recognizing the same epitope but differing in affinity.

Further details of the characterization of these antibodies are provided in the examples below.

Immunochemicals

The immunochemical derivatives of the monoclonal antibodies of this invention that are of prime importance are immunotoxins (conjugates of the antibody and a cytotoxic moiety) and labeled (e.g., radiolabeled, enzyme-labeled, or fluorochrome-labeled) derivatives in which the label provides a means for identifying immune complexes that include the labeled antibody.

The cytotoxic moiety of the immunotoxin may be a cytotoxic drug or an enzymatically active toxin of bacterial or plant origin, or an enzymatically active fragment ("A chain") of such a toxin. Enzymatically active toxins and fragments thereof are preferred and are exemplified by diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from *Pseudomonas aeruginosa*), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, *Aleurites fordii* proteins, dianthin proteins, *Phytolacca americana* proteins (PAPI, PAPII, and PAP-S), momordica charantia inhibitor, curcin, crotin, saponaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, and enomycin. Ricin A chain, nonbinding active fragments of diphtheria toxin, abrin A chain, and PAPII are preferred. Conjugates of the monoclonal antibody and such cytotoxic moieties may be made using a variety of bifunctional protein coupling agents. Examples of such reagents are SPDP, IT, bifunctional derivatives of imidoesters such as dimethyl adipimidate.HCl, active esters such as disuccinimidyl suberate, aldehydes such as glutaraldehyde, bis-azido compounds such as bis(p-azidobenzoyl) hexanediamine, bis-diazonium derivatives such as bis-(p-diazoniumbenzoyl)-ethylenediamine, diisocyanates such as tolylene 2,6-diisocyanate, and bis-active fluorine compounds such a 1,5-difluoro-2,4-dinitrobenzene.

When used to kill human breast cancer cells in vitro for diagnostic purposes, the conjugates will typically be added to the cell culture medium at a concentration of at least about 10 nM. The formulation and mode of administration for in vitro use are not critical. Aqueous formulations that are compatible with the culture or perfusion medium will normally be used. Cytotoxicity may be read by conventional techniques to determine the presence or degree of breast cancer.

When used in vivo for therapy, the immunotoxins are administered to the patient in therapeutically effective amounts (i.e., amounts that eliminate or reduce the patient's tumor burden). They will normally be administered parenterally, preferably intravenously. The dose and dosage regimen will depend upon the nature of the cancer (primary or metastatic) and its population, the characteristics of the particular immunotoxin, e.g., its therapeutic index, the patient, and the patient's history. The amount of immunotoxin administered will typically be in the range of about 0.1 to about 10 mg/kg of patient weight.

For parenteral administration the immunotoxins will be formulated in a unit dosage injectable form (solution, suspension, emulsion) in association with a pharmaceutically acceptable parenteral vehicle. Such vehicles are inherently nontoxic and nontherapeutic. Examples of such vehicles are water, saline, Ringer's solution, dextrose solution, and 5% human serum albumin. Nonaqueous vehicles such as fixed oils and ethyl oleate may also be used. Liposomes may be used as carriers. The vehicle may contain minor amounts of additives such as substances that enhance isotonicity and chemical stability, e.g., buffers and preservatives. The immunotoxin will typically be formulated in such vehicles at concentrations of about 1 mg/ml to 10 mg/ml.

Cytotoxic radiopharmaceuticals for treating breast cancer may be made by conjugating high linear energy transfer (LET) emitting isotopes (e.g., Y, Pr) to the antibodies. The term "cytotoxic moiety" as used herein is intended to include such isotopes.

The labels that are used in making labeled versions of the antibodies include moieties that may be detected directly, such as fluorochromes and radiolabels, as well as moieties, such as enzymes, that must be reacted or derivatized to be detected. Examples of such labels are ^{32}P, ^{125}I, ^{3}H, ^{14}C, fluorescein and its derivatives, rhodamine and its derivatives, dansyl, umbelliferone, luciferia, 2,3-dihydrophthalazinediones, horseradish peroxidase, alkaline phosphatase, lysozyme, and glucose-6-phosphate dehydrogenase. The antibodies may be tagged with such labels by known methods. For instance, coupling agents such as aldehydes, carbodiimides, dimaleimide, imidates, succinimides, bis-diazotized benzadine and the like may be used to tag the antibodies with the above-described fluorescent, chemiluminescent, and enzyme labels.

The antibodies and labeled antibodies may be used in a variety of immunoimaging or immunoassay procedures to detect the presence of breast cancer in a patient or monitor the status of such cancer in a patient already diagnosed to have it. When used to monitor the status of a cancer a quantitative immunoassay procedure must be used. In such monitoring assays are carried out periodically and the results compared to determine whether the patient's tumor burden has increased or decreased. Common assay techniques that may be used include direct and indirect assays. Direct assays involve incubating a tissue sample or cells from the patient with a labeled antibody. If the sample includes breast cancer cells, the labeled antibody will bind to those cells. After washing the tissue or cells to remove unbound labeled antibody, the tissue sample is read for the presence of labeled immune complexes. In indirect assays the tissue or cell sample is incubated with unlabeled monoclonal antibody. The sample is then treated with a labeled antibody against the monoclonal antibody (e.g., a labeled antimurine antibody), washed, and read for the presence of labeled ternary complexes.

For diagnostic use the antibodies will typically be distributed in kit form. These kits will typically comprise: the antibody in labeled or unlabeled form in suitable containers, reagents for the incubations and washings, a labeled antimurine antibody if the kit is for an indirect assay, and substrates or derivatizing agents depending on the nature of the label. Human breast cancer antigen controls and instructions may also be included.

The following examples provide a detailed description of the preparation, characterization, and use of representative monoclonal antibodies of this invention. These examples are not intended to limit the invention in any manner.

Immunization

Fresh postsurgical human breast cancer tissue and a variety of normal tissues were used to prepare membrane extracts by homogenization and discontinuous sucrose gradient centrifugation. Human breast cancer cell lines were obtained from the Breast Cancer Task Force, the American Type Culture Collection (ATCC), and from Dr. Jorgen Fogh at Memorial Sloan Kettering. The cells were maintained and passaged as recommended by the Breast Cancer Task Force, the ATCC and Dr. Fogh. For immunizations, either membrane extract containing 100 μg of protein (Lowry assay) or ten million live breast cancer cells were inoculated intra-peritoneally into five week old Balb/c mice. The mice were boosted identically twice at monthly intervals. Three days after the last boost, the spleens were removed for cell fusion.

Hybridoma Methods

Somatic cell hybrids were prepared by the method of Buck, D. W., et al, supra, using the murine myeloma lin Sp-2/0/Ag14. All hybridoma cell lines were cloned by limiting dilution. Half of the fusions employed splenocytes from mice immunized with breast cancer membrane extracts and half used splenocytes from mice immunized with live breast cancer cell lines. Eighty-three thousand four hundred twenty-four wells were generated from those fusions, of which 22,459 exhibited hybridoma growth.

Screening Methods

Hybridoma supernatant was assayed for reactive antibody in either a sold phase enzyme-linked immunosorbent assay (ELISA) with the immunizing breast cancer membrane extract or an indirect immunofluorescence assay with the immunizing breast cancer cell line. For the sold phase membrane ELISA, 40 μl of 0.1 mg/ml breast cancer membrane protein were placed in polyvinyl chloride (PVC) microtiter wells for 12 hr at 4° C. The extract was aspirated and the wells washed with phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA). The wells were then incubated with 45 μl of a 1:10 dilution of hybridoma supernatant. The diluent was media with 25 mM of a buffer, 10% bovine serum, and 0.1% sodium azide. After 30 min at room temperature, the wells were again washed and incubated 45 min at 37° C. with a 1:200 dilution of peroxidase conjugated goat anti-mouse IgG. The dilutent was PBS. The wells were then washed with PBS and reacted with 200 μl of 2,2-azino-di(3-ethylbenzthiazoline sulphonic acid) in 0.1M sodium citrate buffer pH 4.2 for 30 min at room temperature. Optical density was measured at 405 nm on a Micro-Elisa Reader. For each experiment a positive control, anti-beta 2 microglobulin at 5 μg/ml, was reacted with normal human kidney membrane. This gave an optical density of 1.0 ± 0.1 (standard deviation). The background was 0 ± 0.1 optical density units (O.D.) using media without mouse monoclonal antibody. Wells that gave a reaction on the breast cancer membrane extract of greater than 0.7 O.D. were saved.

For the indirect immunofluorescence cell line assay we placed one hundred thousand breast cancer cells of the immunizing cell line overnight with appropriate media in each chamber of a set of eight chambered slides. Similarly, one hundred thousand fibroblast cells from cell line CC95 were incubated overnight in chambered slide wells. The cells were washed with PBS containing 1% BSA. The wells, both breast cancer and fibroblast, were incubated for 30 min at 4° C. with 1:10 dilutions of hybridoma supernatant. The cells were again washed and incubated 30 min at 4° C. with a 1:50 dilution of fluorescein isothiocyanate (FITC)-conjugated goat $F(ab')_2$ anti-mouse Ig. The cells were washed three times, fixed in 1.5% formaldehyde in PBS for five min, chambers removed and rinsed in PBS. The slides were then mounted in a composition containing polyvinyl alcohol, glycerol, biffers and a preservative and examined with a fluorescence microscope. Hybridoma wells showing strong fluorescent binding to breast cancer cells but no fluorescent binding to fibroblasts were saved. Five thousand one hundred fifty-six hybridoma

wells revealed breast cancer reactivity in the initial screen.

Supernatants from the 5156 positive wells were then tested in solid phase ELISA with eight normal tissue membrane extracts (liver, lung, colin, stomach, kidney, tonsil, spleen and pancreas). Any well supernatant giving an ELISA O.D. greater than 0.3 was discarded. One thousand one hundred one of the supernatants were found to be unreactive with the normal tissue extracts.

The 1101 hybridoma supernatants were tested on frozen sections of human breast carcinoma tissues. Six micron sections were attached to slides, fixed 10 min in acetone at 4° C., dried 10 min at room temperature, washed with PBS, blocked with horse serum and incubated 20 min at room temperature with 200 μl neat hybridoma supernatant. The slides were washed with PBS, and finally incubated 20 min at 37° C. with a 1:50 dilution of peroxidase conjugated rabbit anti-mouse Ig, washed again with PBS, and finally incubatged 7.5 min at 37° C. with 0.5 mg/ml diaminobenzidine in 0.05M Tris buffer pH 7.2 containing 0.01% hydrogen peroxide. The slides were stained with hematoxylin, dehydrated and mounted in a medium containing 35.9% methyl/n-butylmethacrylate copolymer, 7.1% butyl benzyl phthalate, and 0.3% 2,6-ditertbutyl-p-cresol. One hundred twenty-four wells yielded breast cancer selective binding and were cloned.

Purification and Class Determination

Immunoglobulin class and subclass of the monoclonal breast cancer selective antibodies were determined. Antibodies were also internally labeled by growing $2–3\times10^6$ hybridoma cells for 4 hr in methionine-free medium containing 0.2 μCi ^{35}S methionine. ^{35}S-labeled antibodies were immunoprecipitated with fixed staphylococcus A cells, or with the composition used to fix the staphylococcus A cells precoated with rabbit anti-mouse immunoglobulin, and the immunoprecipitates were analyzed by SDS-PAGE to determine antibody light and heavy chain mobility, lack of extra chains, and the ability of each antibody to bind staphylococcal protein A.

The antibodies were expanded in vivo. Balb/c or Fl (C57B/6×Balb/c) mice were primed with 0.5 ml pristane intraperitoneally (ip) and after 10–14 days inoculated with one million long phase hybrodima cells in PBS. Ascites fluid was stored at −70° C. and thawed and filtered through a 0.8 micron filter unit before further purification.

IgG antibodies that bound staphylococcal protein A were purified by affinity chromatography on protein A-chromatographic resin containing agarose, dextron and/or acrylamide with pH step gradient elution. IgG antibodies that did not bind protein A were precipitated by addition of ammonium sulfate to 40% saturation at 0° C. The precipitated were redissolved in PBS, dialysed to 20 mM Tris pH 7.2 and chromatographed on a 1.6×50 cm column of diethylaminoethyl cellulose (DEAE) eluting with a 1.5 liter 0–600 mM NaCl gradient at 4° C. at a flow rate of 1 ml/min. In each case, column fractions were monitored by SDS-PAGE and the purest antibody fractions were pooled, concentrated to 1–3 mg/ml, dialysed to PBS/0.02% NaN_3, and stored at 4° C.

IgM antibodies were purified by gel filtration on a 2.6×40 cm column of chromatographic resin containing agarose, dextron and/or acrylamide eluting with PBS/0.01% sodiumn azide at room temperature at a flow rate of 1 ml/min.

Selectivity Determination

In order to evaluate their selectivity for breast cancer, the purified antibodies were tested by immunoperoxidase section staining on sections of sixteen normal tissues, and by immunofluorescent cell sorting on five blood cell types. Immunoperoxidase staining was performed as above except that known dilutions of purified antibodies in PBS in the range of 1–40 μg/ml were used instead of hybridoma supernatants. The pure antibodies were first titrated to find the minimal concentration giving strong immunoperoxidase staining on breast cancer sections and then used at that concentration for the normal tissue tests. Peripheral blood cells (platelets, lymphocytes, red blood cells, granulocytes, and monocytes) were prepared by centrifugation using a medium which separates monocytes from polymorphonuclear leucocytes. The cells were reacted with antibody at the optimal concentration determined above for 30 min at 4° C., washed, reacted with a 1:50 dilution of fluorescence isothiocyanate-conjugated goat anti-mouse Ig for 30 min at 4° C., washed again and examined in a cell sorter. The wash buffer and diluents were PBS with 1% gelatin and 0.02% sodium azide. The cell sorter was equipped with a 76 micron nozzle and a one watt argon ion laser at 488 nm. An 80 mm confocal lens was used on the optical rail assembly for focusing. Other filters used were a 515 nm interference filter and a 515 nm absorbance filter (for scattered laser light) and a neutral density 1.5 filter for forward angle light scatter. Contour plots of log fluorescein fluorescence versus forward angle light scatter were used for sample analysis.

The binding behaviors of the claimed antibodies are reported in Table I below.

TABLE I

ANTIBODY BINDING TO NORMAL ISSUE SECTIONS

	Tissue															
Antibody	Pancreas	Esophagus	Lung	Kidney	Colon	Stomach	Brain	Tonsil	Liver	Heart	Ovary	Skin	Breast	Bone	Uterus	Bladder
33F8	0	2E	0	1T	0	0	0	1Ly	0	0	0	1W	0	1Mk	1L	1E
113F1	2Ac	2E	0	0	0	2G	0	1E	0	0	0	0	0	0	0	1E
245E7	1L	0	1A, M	0	0	2L	0	1E	0	0	0	2S	2L	0	2L	1E
2G3	2Ac	2E	1A	2T	0	1L	0	1E	0	0	0	0	2E	0	2L	2E
260F9	1Ac	2E	0	1T	0	1G	0	2E	2D	0	0	2E, 2H	2E	0	1L	2E
280D11	0	1E	0	2T, 2B	1L	2L	0	0	2D	0	0	1E, 1H	2L	2Gr	2G	0
266B2	1Ac, 1D	2E	0	1T	0	0	0	2E	0	0	0	2E, 2W	1E	0	0	1E
454C11	1D	1-2E	0	1T	0	0	0	1E	1D	0	0	1E, H	1E	0	1G	1E
317G5	1Ac, I	0	0	2T	1G	0	0	0	2D	0	0	0	0	0	1G	0
520C9	0	0	0	1T	0	0	0	0	0	0	0	0	0	0	0	0
452F2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

TABLE 1-continued

ANTIBODY BINDING TO NORMAL ISSUE SECTIONS

Antibody	Tissue															
	Pancreas	Esophagus	Lung	Kidney	Colon	Stomach	Brain	Tonsil	Liver	Heart	Ovary	Skin	Breast	Bone	Uterus	Bladder
369F10	0	0	0	0	0	1G	0	0	0	0	0	1S	0	0	0	0
736G9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
741F8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
758G5	0	0	0	1T	0	0	0	0	0	0	0	0	0		0	0
761B10	0	0	0	1T	0	0	0	0	0	0	0	0	0		0	0

Staining intensity: 2 = strong; 1 = weak; 0 = negative.
A = alveolar cells; Ac = acini; B = Bowman's capsule; D = ducts; E = epithelial; G = glands; Gr = granulocytes; H = hair follicles; I = islets; L = lumen ± apical cytoplasm; Ly = lymphocytes; M = macropnages; Mk = megakonyocytes; My = myelin; S = sepaceous; St = stroma; T = tubules; U = glomeruli; W = sweat glands. There was no binding to platelets, red cells, lymphocytes, monocytes or granulocytes except 280D11 weakly binding granulocytes. None of the antibodies bound fibroblasts.

Range Determination

In order to determine how wide a range of breast cancers might be recognized by each antibody, the breast cancer selective antibodies were tested by immunoperoxidase staining on frozen sections of 27 different breast tumors. The breast cancers used for section staining were all infiltrating intraductal carcinomas, so no correlation of antibody binding with histologic type of breast cancer could be made. In addition, no correlation between antibody binding and the nodal status or estrogen receptor status was found for the twelve tumors for which donor information was available. Antibodies reacted equally well with metastatic and primary breast tumors. The results of these tests for the claimed antibodies are reported in Table 2 below.

TABLE 2

ANTIBODY BINDING TO BREAST CANCER TISSUE SECTIONS*

Antibody	Breast Cancers													
	LA	KA	JA	IA	HA	GA	E	EA	TA	UA	RA	SA	O	R
2G3	1	2	1	2	2	2	ND	2	2	2	2	2	2	2
33F8	1	1	0	0	0	0	1	0	0	0	0	0	1	0
113F1	0	1	1	0	0	0	0	0	0	0	1	0	0	0
245E7	2	2	2	2	2	2	2	2	2	2	2	2	2	2
260F9	0	1	0	1	0	1	ND	1	2	0	0	0	1	0
280D11	2	2	0	1	2	1	ND	1	1	0	1	0	1	1
266B2	1	2	0	1	0	1	ND	0	2	1	1	0	1	0
454C11	1	2	0	2	1	1	ND	2	1	1	0	0	ND	0
317G5	1	ND	0	0	1	ND	ND	0	0	0	1	1	ND	0
520C9	0	ND	0	2	0	ND	ND	2	0	1	0	0	ND	0
452F2	0	2	0	2	0	0	ND	1	0	1	0	0	ND	0
369F10	2	2	2	2	0	1	ND	1	0	1	1	2	2	0
736G9	2	ND	0	2	0	ND	ND	1	0	1	0	0	ND	0
741F8	0	ND	0	2	0	ND	ND	2	0	1	0	0	0	0
758G5	1	ND	0	0	0	ND	ND	2	0	1	0	0	ND	0
761B10	1	ND	0	2	0	ND	ND	2	0	1	0	0	ND	0

Antibody	Breast Cancers												
	MA	BA	NA	FA	LMA	LME	MBA	Z	YA	KB	CB	IC	EC
2G3	1	2	2	2	2	2	2	2					
33F8	0	0	0	0	0	0	0	ND					
113F1	0	0	0	0	0	0	0	ND					
245E7	2	2	2	2	2	2	2	ND					
260F9	0	1	1	1	0	1	1	0					
280D11	1	1	0	1	1	1	1	1					
266B2	1	1	2	1	0	1	1	1					
454C11	2	0	0	1	0	1	1	ND					
317G5	1	0	1	1	0	0	0	0	0	1	1		
520C9	2	0	1	0	0	0	0	0					
452F2	1	0	0	0	0	0	0	0					
369F10	0	2	2	2	2	2	2	1					
736G9	1	0	0	0	0	0	0	0	0	0	0		
741F8	2	0	0	0	0	0	0	0	0	0	0		
758G5	2	0	0	1	0	0	0	0	1	0	1		
761B10	2	0	0	0	0	0	0	0	0	0	1		

*Staining intensity: 2 = strong; 1 = weak; 0 = negative; ND = not determined.

Antibodies were further evaluated for range of breast cancer recognition by cell line immunofluorescence assays on 14 breast cancer cell lines. Table 3 below reports the results of these tests for the claimed antibodies.

TABLE 3

ANTIBODY BINDING TO BREAST CANCER CELL LINES*

Antibody	SKBr3	BT483	MCF7	BT20	ZR751	MDAMB231	CAMA1
2G3	+	+	+	+	+	+	+
33F8	+	+	+	+	+	−	+

TABLE 3-continued

ANTIBODY BINDING TO BREAST CANCER CELL LINES*							
113F1	+	+	+	+	+	+	+
245E7	+	+	+	+	+	+	+
260F9	+	+	+	+	+	+	+
280D11	+	+	+	+	+	−	+
266B2	+	+	+	+	+	+	+
454C11	+	+	+	+	+	+	+
317G5	+	+	+	+	+	−	+
520C9	+	+	−	−	−	NT	+
452F2	+	+	−	−	+	NT	+
369F10	−	+	−	−	−	−	+
736G9	+	+	−	NT	NT	NT	+
741F8	+	+	−	NT	NT	NT	+
758G5	+	+	−	NT	NT	NT	−
761B10	+	+	−	NT	NT	NT	−
Antibody	ALAB	BT549	BT474	T47D	MDAMB157	MDAMB330	ZR7530
2G3	+	+	+	+	+	−	+
33F8	+	+	+	−	+	+	−
113F1	−	−	+	+	+	+	−
245E7	+	+	+	+	+	−	+
260F9	+	−	+	+	+	+	+
280D11	−	+	+	+	+	−	+
266B2	−	−	+	+	−	+	+
454C11	+	−	NT	−	NT	NT	+
317G5	+	−	NT	+	+	−	+
520C9	NT	−	NT	−	NT	NT	+
452F2	+	−	NT	−	NT	NT	+
369F10	−	−	NT	−	NT	NT	−
736G9	NT	−	NT	+	NT	NT	+
741F8	NT	−	NT	+	NT	NT	+
758G5	NT	−	NT	−	NT	NT	+
761B10	NT	−	−	NT	+	NT	+

*Cell line binding: + = positive; − = negative; NT = not tested.

Finally, the antibodies were tested by immunoperoxidase staining on eleven non-breast malignancies. The results for the claimed antibodies are reported in Table 4 below.

TABLE 4

	ANTIBODY BINDING TO CANCERS*										
Antibody	Colon	Lung	Prostate	Pancreas	Uterine	Lymphoma	Stomach	Bladder	Esophagus	Melanoma	Ovarian
2G3	2	0	2	0	2	0	2	0	0	2	2
33F8	0	1	0	0	1	0	0	0	0	0	1
113F1	0	2	0	2	1	2	2	0	1	0	0
245E7	0	2	2	2	2	2	0	0	0	0	2
260F9	0	0	1	1	1	0	0	0	1	0	2
266B2	0	1	1	1	1	0	1	0	1	0	1
280D11	0	0	1	1	1	2	0	0	0	0	2
454C11	0	0	1	1	2	0	0	0	0	0	1
317G5	1	1	0	0	1	0	0	0	0	0	0
520C9	0	1	1	1	1	0	0	0	0	0	0
452F2	0	0	0	0	0	0	0	0	0	0	0
369F10	0	1	1	1	0	0	0	0	0	0	2
736G9	0	0	0	0	0	0	0	0	0	0	0
741F8	0	0	0	0	0	0	0	0	0	0	0
758G5	0	1	0	0	0	0	0	0	0	0	0
761B10	0	0	0	0	0	0	0	0	0	0	0

*Staining intensity: 2 = strong; 1 = weak; 0 = negative. Only one tumor of each type examined.

Cytotoxicity Evaluation

The claimed antibodies were conjugated to ricin toxin A chain (RTA) treated with SPDP as described in Carlsson, J., et al, Biochem J (1978) 173: 723–737 or with iminothiolane (IT).

SPDT Conjugation

SPDP (20 mM in ethanol) was added in a 20-fold molar excess to antibody and following a 30 min incubation at room temperature, the unreacted SPDP was removed by dialysis against PBS. The extent of derivatization was determined by measuring the release of pyridine-2-thione at 343 nm after reduction with dithiothreitol (DTT). Depending on the antibody, three to eight lysine amino acid groups (per antibody molecule) were converted to the pyridyl-disulfide derivative.

The SPDP-treated antibodies were conjugated with RTA. Immediately prior to conjugation, the RTA was reduced with 50 mM DTT, then desalted on a column of chromatographic resin containing agarose, dextran and/or acrylamide to remove DTT from protein. Reduced RTA was added in a three- to five-fold molar excess over pyhridyl-disulfide antibody. A typical reaction mixture (1 ml) consisted of 7 μmM antibody and 30 μm RTA. The reaction was allowed to proceed overnight at 4° C. The extent of conjugation of RTA to antibody was determined spectrophotometrically by measuring the release of pyridine-2-thione. On the aver-

age, conjugates contained two to three RTA molecules per antibody molecule. This was confirmed by nonreducing SDS-PAGE gels (7.5%), which also revealed that the typical conjugate preparation contained 10%–30% free antibody.

The conjugate mixture was chromatographed on an HPLC size exclusion column to separate conjugates from residual unreacted RTA. The column was equilibrated in 0.1 sodium sulfate/0.02M sodium phosphate pH 6.8. Conjugate mixture (0.7 ml) was injected, then chromatographed at a flow rate of 1 ml/min (room temperature). Fractions of 0.5 ml were collected and the peak conjugate fractions were pooled and filter sterilized prior to cytotoxicity testing.

Iminothiolane Conjugation

Approximately 30 mg/ml antibody in 0.10M Na phosphate, 0.001M Na EDTA, pH 8.0 (hereafter referred to as P-EDTA buffer) is reacted with 1 mM 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) at room temperature for about 15 min and then chilled to 0° C. in an ice bath. Enough IT is added to this solution to give 2.5 IT molecules/antibody molecule, and the resulting solution is stored at 0°–5° C. against three 100-fold excess volumes of P-EDTA buffer.

RTA, normally stored in P-EDTA containing 1 mM DTT, is ultrafiltered to a concentration between 10 and 15 mg/ml and dialyzed at 0°–5° C. against three 100-fold excess volumes of P-EDTA. Enough RTA is added to the derivatized antibody to give 1.0–1.2 free thiols on RTA/blocked thiol on derivatized antibody. This mixture is incubated at room temperature for 2 hr.

The coupling reaction mixture is applied to a column of a chromatographic resin based on a blue dye hooked up to a solid support, which mixture is then eluted with P-EDTA at room temperature. The column is scaled to contain approximately 2 ml of bed volume per mg of starting antibody. After an initial peak of unconjugated antibody has been eluted from the column, the eluant is switched to P-EDTA containing 1M NaCl. Immunoconjugate and unreacted RTA are eluted in this buffer as a very sharp peak, which is pooled and dialyzed at 0°–5° C. against one 10-fold excess volume of 0.15M Na phosphate, pH 7.1 (hereafter referred to as p_i buffer). The dialyzed protein is applied to a column of a gel at 0°–5° C. and eluted with buffer at a flow rate of 6 cm/hr. The column is scaled to contain at least 25 ml of bed volume/ml of applied protein. Immunoconjugate is eluted as a single peak, slightly after the excluded volume, baseline-resolved from following peaks of dimerized and monomeric RTA. TGhe pooled immunoconjugate peak is ultrafiltered at 35 psi to a final concentration of 5.0 mg/ml and filter-sterilized.

Cytotoxicity Texts

The test human breast cancer lines used in the cytotoxicity tests were MCF-7, CAMA-1, SKBR-3, and BT-20. The human fibroblast cell lines CC95 and WI-38 were used as negative controls.

Forty thousand test cells in 1 ml medium were added to a set of 8 ml glass vials, followed by the addition of conjugate dilutions (in PBS containing 100 μg/ml BSA). After incubation at 37° C. for 22 hr, the medium was aspirated, the monolayers were washed with PBS, and methionine-free medium supplemented with ^{35}S methionine was added. The vials were further incubated 2 hr at 37° C., the medium was removed, and the cells were washed twice with 2 ml of 10% trichloroacetic acid containing 1 mg/ml methionine. The cells were dried, scintillation fluid was added, and the radioactivity was counted in a scintillation counter. Cytotoxicity was expressed as the tissue culture inhibitory dose of conjugate that resulted in 50% of control (untreated) protein synthesis (TCID 50%).

The results of these cytotoxicity tests are reported in Table 5 below.

TABLE 5

RTA Conjugate	CYTOTOXICITY OF BREAST TUMOR IMMUNOTOXINS TCID 50% (nM)						
	Isotype	MCF-7	CAMA-1	SKBR-3	BT-20	CC95	WI-38
260F9	G1	0.1	0.4	0.06	9	>50	>50
317G5	G1	0.4	5	10	2	>50	>50
113F1	G3	0.5	0.6	10	6	>50	>50
2G3	G1	0.8	1	>50	15	>50	ND
266B2	G1	1	5	0.5	10	>50	ND
280D11	G1	1	1	0.9	>40	>50	>50
245E7	G1	6	8	8	4	>50	>50
454C11	G2a	6	>20	0.3	30	≧50	≧50
33F8	G1	10	ND	ND	ND	ND	ND
369F10	M	10	ND	ND	ND	ND	ND
520C9	G1	>50	>50	10	>50		
452F2	G1	20		10			
736G9	G1	>50	>50	1.3	>50		
741F8	G1	>80	>80		>80		
758G5	G1	>50		0.3			
761B10	G1	>50		1.0			

ND = not determined.

In vivo Testing of Conjugates

Conjugates of 245E7, 280D11, and 260F9 with RTA were made as above using iminothiolane or SPDP as a coupling agent. The efficacies of these conjugates against MX-1 human breast tumor cells in vivo was evaluated as follows.

Female athymic Balb/c-nu/nu mice (20–24 g) were used. Fragments, 1.0 mm^3, were obtained from 600–800 mm^3 tumors with no signs of central necrosis and packed into a syringe. Mice were implanted s.c. with 0.05 ml of the suspension in the axillary region with mediolateral puncture. On day 7 or 14 after implant the mice were weighed and their tumor burdens were evaluated by measuring the implants with calipers. Mice were grouped according to mean tumor size.

The conjugates were injected i.v. into the tail vein of control mice Q2D×6 to determine the maximum tolerable dose of the particular conjugate. Based on these results, dose regimens for administering the conjugates

to tumor-bearing mice were selected. Groups of tumor-bearing mice and control mice were injected i.v. with the conjugates according to the chosen regimens. Animal reactions, side effects, and mortalities were monitored daily along with tumor volume and animal weight measurements. Changes in tumor volume at the end of the test period were calculated based on the average of the sum of measurements over the test period. The results of these tests are reported in Table 6 below.

TABLE 6

Conjugate	LD_{50} (μg/m)	Dose/Schedule	Tumor Age/Volume (6–10 mice/gp)	% MX1-Tumor Growth Inhibitions	FBW / IBW
245E7-SPDP-RTA	410	125 μg iv/god × 6	18d (300–400)	74.8 (D14) only 3 animals	1.15
245E7-IT-RTA	350	200 μg iv/god × 5	14d (100–200)	62.5 (D14) $p < 0.05$	0.93
280D11-IT-RTA	350	200 μg iv/god ×5	14d (100–200)	70.0 (D13) $p < 0.01$[1]	0.99
		200 μg iv/god × 4	6d (25–50)	80.0 (D14) $p < 0.02$	0.97
260F9-IT-RTA	400	200 μg iv/god ×5	14d (100–200)	20.3 (D14) NS[2]	1.02
		100 μg iv/god × 3–4	14d (100–200)	17.6 (D14) NS[3]	1.01
245E7-IT-RTA + 280D11-IT-RTA (cocktail)		200 μg iv/god × 5	14d (100–300)	70.0 (D14) $p < 0.01$ 80.0 (D10) $p < 0.001$	1.00 0.91

[1]Regression 60.5% (D6) $p < 0.001$ 0.84
[2]50.8% (D11) $p < 0.05$ 0.98
[3]44.8% (D11) $p < 0.1$ 0.87
NS = not significant
D = days after initiation of treatment

Antibody Affinity and Antigen Density

Several of the claimed antibodies were iodinated and tested for binding to MCF-7 or BT-20 cells. The antibodies were labeled with ^{125}I using chloramine T to a specific activity of approximately 10 μCi/μg. To determine immunoradiochemical purity, 100,000 cpm of two of the labeled antibodies in 0.5 ml fetal calf serum was serially absorbed with five aliquots of target cells for 15 min at 0° C. (generaly 4,000,000 MCF-7 breast cancer cells per aliquot), and the remaining radioactivity in the supernatant after each absorption was determined.

For measurements of association constants, known concentrations of labeled and unlabeled monoclonal antibodies were incubated with target cells in fetal calf serum for 15 min in ice. Aliquots of the cell/antibody mix were then counted in a gamma counter or filtered through Microfold filter plates (V & P Scientific) and the filters counted. To account for unbound antibody retained in liquid on the filters, controls containing the same concentrations of antibody but no cells were done in parallel. Association constants and antigen copy number per target are calculated from the affinity test results and are reported in Tabled 7 below.

TABLE 7

Antibody	n	Ka	nKa
2G3	3.7e6	9.1e6	3.4e13
113F1	2.3e6	1.1e9	2.5e15
260F9	3.1e5	5.6e7	1.7e13
266B2	8.0e4	2.7e8	2.2e13
280D11	3.9e5	8.8e6	3.4e12
317G5	3.2e6	1.6e6	5.1e12
452F2	2.5e5	6.8e6	1.7e12
454C11	3.9e5	4.8e7	1.9e13
520C9	5.0e5	8.2e6	4.1e12

n = the antigen copy number per MCF-7 cell; Ka = association constant on MCF-7. nKa is the product of n and Ka and relates antibody concentration to antibody bound per cell.

Immunoprecipitation tests on the antibodies indicated that seven of them (454C11, 452F2, 520C9, 736G9, 741F8, 758G5, and 761B10) bind a common monomeric c.a. 210,000 dalton protein found in cancerous breast tissue. Six of the seven (452F2, 520C9, 736G9, 741F8, 758G5, and 761B10) are believed to recognize the same epitope on the 210,000 dalton protein. Of these six, relative affinity studies indicated that 520C9 had the highest association constant.

Samples of the hybridomas that produce the claimed monoclonal antibodies were deposited in the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Md. 20852-1776, USA. Only 520C9 was deposited of the six hybridomas that produce antibody that recognizes the same epitope on the 210,000 dalton protein. The five that were not deposited are considered to be functionally equivalent to 520C9. Their ATCC accession numbers and deposit dates for the deposited hybridomas are:

Hybridoma/ Antibody Designation	Deposit Date	Accession No.
260F9	January 27, 1984	HB 8488
113F1	January 27, 1984	HB 8490
2G3	January 27, 1984	HB 8491
280D11	January 27, 1984	HB 8487
266B2	January 27, 1984	HB 8486
245E7	January 27, 1984	HB 8489
454C11	January 27, 1984	HB 8484
33F8	January 9, 1985	HB 8697
317G5	January 27, 1984	HB 8485
520C9	January 8, 1985	HB 8696
369F10	December 13, 1984	HB 8682
*260F9-1C9	November 7, 1984	HB 8662.

*This clone is a progeny of 260F9 and was found to be a better antibody producer than 260F9.

These deposits were made under the Budapest Treaty and will be maintained and made accessible to others in accordance with the provisions thereof.

We claim:

1. A murine monoclonal antibody that:
(a) binds selectively to human breast cancer cells;
(b) has a G or M isotype;
(c) when conjugated to ricin A chain, exhibits a TCID 50% of less than about 10 nM against at least one of MCF-7, CAMA-1, SKBR-3, or BT-20 cells; and
(d) binds a human breast cancer antigen that is also bound by a reference antibody selected from the group consisting of 260F9, 113F1, 266B2, 454C11, 33F8, 317G5, 520C9, and 260F-9-1C9, as determined by immunoprecipitation or sandwich immunoassay.

2. The monoclonal antibody of claim 1 wherein said reference antibody is selected from the group consisting of 260F9, 266B2, 113F1, 454C11 and 317G5.

3. A murine x murine hybridoma cell line that produces the monoclonal antibody of claim 2.

4. An immunotoxin comprising a conjugate of (a) the monoclonal antibody of claim 2; and (b) a cytotoxic moiety.

5. The monoclonal antibody of claim 2, wherein said antibody is labeled with a detectable label.

6. A method of diagnosing whether a human cell is a breast cancer cell comprising

(a) incubating a human cell with the antibody of claim 5; and

(b) determining the presence of labeled binary immune complexes on the human cell.

7. A method of diagnosing whether a human cell is a breast cancer cell comprising:

(a) incubating said human cell with the monoclonal antibody of claim 2;

(b) incubating the human cell with a labeled antibody against said monoclonal antibody;

(c) determining the presence of labeled ternary immune complexes on the human cell.

8. The monoclonal antibody of claim 1 wherein said reference antibody is 260F9 or 266B2.

9. The monoclonal antibody of claim 1 wherein said reference antibody is 454C11.

10. The monoclonal antibody of claim 9 wherein said antibody is labeled with a detectable label.

11. A method of diagnosing whether a human cell is a breast cancer cell comprising

(a) incubating a human cell with the antibody of claim 10; and

(b) determining the presence of labeled binary immune complexes on the human cell.

12. A method of diagnosing whether a human cell is a breast cancer cell comprising:

(a) incubating said human cell with the monoclonal antibody of claim 9;

(b) incubating the human cell with a labeled antibody against said monoclonal antibody;

(c) determining the presence of labeled ternary immune complexes on the human cell.

13. The monoclonal antibody of claim 1 wherein said reference antibody is 317G5.

14. The monoclonal antibody of claim 1 wherein said reference antibody is 113F1.

15. The monoclonal antibody of claim 1 wherein said reference antibody is 33F8.

16. The monoclonal antibody of claim 1 that binds the same epitope as the reference antibody.

17. The monoclonal antibody of claim 1 that is 260F9.

18. The cell line of claim 17 selected from the group consisting of 260F9, 113F1, 266B2, 454C11, 33F8, 317G5, 520C9, and 260F91C9.

19. The monoclonal antibody of claim 1 that is 260F9-1C9.

20. The monoclonal antibody of claim 1 wherein the exhibited TCID 50% is less than about 1 nM.

21. A murine x murine hybridoma cell line that produces the monoclonal antibody of claim 1.

22. An immunotoxin comprising a conjugate of

(a) the monoclonal antibody of claim 1 and

(b) a cytotoxic moiety.

23. The immunotoxin of claim 22 wherein the cytotoxic moiety is ricin A chain, PAPII, abrin A chain or a nonbinding, active fragment of diphtheria toxin.

24. A method of killing human breast cancer cells comprising contacting said cells with a cytocidally effective amount of the immunotoxin of claim 23.

25. The immunotoxin of claim 22 wherein the cytotoxic moiety is ricin A chain.

26. A method of killing human breast cancer cells comprising contacting said cells with a cytocidally effective amount of the immunotoxin of claim 22.

27. The monoclonal antibody of claim 1 wherein said antibody is labeled with a detectable label.

28. A method of diagnosing whether a human cell is a breast cancer cell comprising

(a) incubating a human cell with the antibody of claim 27 and

(b) determining the presence of labeled binary immune complexes on the human cell.

29. A method of diagnosing whether a human cell is a breast cancer cell comprising:

(a) incubating said human cell with the monoclonal antibody of claim 21;

(b) incubating the human cell with a labeled antibody against said monoclonal antibody;

(c) determining the presence of labeled ternary immune complexes on the human cell.

* * * * *

Exhibit 2

United States Patent [19]

Ferris et al.

[11] **Patent Number: 4,771,128**

[45] **Date of Patent: Sep. 13, 1988**

[54] **METHOD OF PURIFYING TOXIN CONJUGATES USING HYDROPHOBIC INTERACTION CHROMATOGRAPHY**

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[21] Appl. No.: **917,469**

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[52] **U.S. Cl.** **530/417**; 530/412; 530/413; 530/391; 530/388; 530/351; 530/387; 530/370; 424/85; 435/68

[58] **Field of Search** 530/412, 387, 417, 391, 530/413, 388; 424/85

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[57] **ABSTRACT**

A method of isolating and purifying toxin conjugates using hydrophobic interaction chromatography. Crude conjugate mixtures are sized to remove unconjugated toxin, and loaded onto a column filled with a suitable hydrophobic gel. Elution is effected with salt solutions of decreasing ionic strength, which salt solutions optionally include increasing amounts of an organic solvent. Toxin conjugate substantially free of unconjugated Ig and unconjugated toxin is provided.

17 Claims, No Drawings

METHOD OF PURIFYING TOXIN CONJUGATES USING HYDROPHOBIC INTERACTION CHROMATOGRAPHY

FIELD OF THE INVENTION

This invention relates generally to chromatographic purification of toxin conjugates, and more particularly relates to a novel method of isolating and purifying immunoconjugates having hydrophobic interaction chromatography.

DESCRIPTION OF THE PRIOR ART

Conjugation of antibodies to toxic drugs and proteins in order to selectively kill tumor cells is an area of research that has recently become of some interest. To a large extent, this is due to the relatively recently developed ability to produce monoclonal antibodies using hybridoma technology, which antibodies are highly specific and can recognize tumor-associated antigens. "Immunoconjugates" may be prepared by covalently linking these antibodies to any of a number of cytotoxic agents. By conjugation, the affinity of the toxins for particular types of tumor cells is increased and the toxins can then exert their effects selectively, by virtue of the specific antibody carriers, against those cells.

Attention has specifically be focused on the highly toxic ribosome-inactivating proteins such as ricin (*Ricin communis,* extracted from castor beans). Preparation of immunoconjugates using these proteins is described, inter alia, in Miyazaki, H., *Gann* 71: 766–774 (1980) and Lambert, J., *J. Bio. Chem.* 260 (22): 12035–12041 (1985)). Ricin consists of two sub-units, termed "A-" and "B-" chains, which are linked by a single disulfide bond. While the A-chain has been shown to be solely responsible for cell death by catalytic inactivation of ribosomes, the B-chain has been demonstrated to provide a binding function, i.e., that chain is able to bind to cell-surface carbohydrates and thus promote the uptake of the A-chain into cells. In order to prepare a suitable immunoconjugate from ricin, then, it is necessary to bind the ricin A-chain to a specific cell-surface binding carrier such as an immunoglobulin (Ig).

Such Ib/ricin A-chain immunoconjugates are known (see, e.g., Miyazaki, supra). Isolation and purification of these immunoconjugates has, however, proved difficult. In prior art methods, while some amount of unreacted A-chain has been removed from the conjugation mixture, unreacted Ig remains in solution, contaminating the immunoconjugate preparation. Other investigators have succeeded in preparing a purified Ig/ricin immunoconjugate; however, that purification process necessitates a multi-step procedure including an ion exchange step (see Lambert, supra). The latter system further requires modification of pH and ionic strength for each conjugate. The present invention is directed to a more versatile and straightforward method of purifying immunoconjugates, which method removes substantially all unreacted antibody and protein from the conjugation mixture. The method uses hydrophobic interaction chromatography as the isolation and purification technique.

Hydrophobic interaction chromatography is a separation technique in which substances are separated on the basis of differing strengths of hydrophobic interaction with an uncharged bed material containing hydrophobic groups. Typically, the column is first equilibrated under conditions favorable to hydrophobic binding, e.g., high ionic strength. As the sample is eluted, a descending salt gradient is applied.

SUMMARY OF THE INVENTION

Accordingly, it is an object of the present invention to provide a method of isolating and purifying toxin conjugates using hydrophobic interaction chromatography.

It is another object of the present invention to provide a straightforward and versatile method of removing unreacted Ig from a crude conjugate mixture.

It is still another object of the invention to provide a method of isolating and purifying immunoconjugates purified by hydrophobic interaction chromatography, which immunoconjugates are substantially free of unreacted Ig and have high specific activities.

Additional objects, advantages and novel features of the invention will be set forth in part in the description which follows, andin part will become apparent to those skilled in the art on examination of the following, or may be learned by practice of the invention.

In one aspect of the present invention, immunoconjugates are prepared using known techniques. In preparing these immunoconjugates, monoclonal antibodies of the IgG class produced by hybridoma cells are linked by a disulfide bridge to a ribosome-inactivating or otherwise cytotoxic protein. The conjugate mixture, which contains unconjugated Ig and protein, including cytotoxic protein, as well as the immunoconjugate, is then purified by at least one chromatographic step. This process involves first removing unconjugated protein via sizing chromatography, followed by hydrophobic gel chromatography. In this latter step, the conjugate mixture is loaded onto a column packed with a gel containing hydrophobic groups, which column is capable of selectively retaining materials of different hydrophobic strengths. The individual components of the conjugate mixture are removed by eluting with salt solutions of decreasing ionic strength and, optionally, increasing amounts of a suitable organic solvent. Immunoconjugate substantially free of both unreacted Ig and unreacted cytotoxic protein is provided.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to a novel method of preparing toxin conjugates substantially free of unconjugated protein. This method, in contrast to known methods, is particularly useful in removing unconjugated Ig and unconjugated protein from crude immunoconjugate mixtures.

Immunoconjugates which may be purified by the novel method include ribosome-inactivating proteins such as ricin A linked by a disulfide bridge to an Ig. Other protein toxins may also be linked by similar disulfide bridges, or thioether linkages in some instances, to form the immunoconjugate. Such other toxins include bacterial toxins, e.g., Pseudomonas exotoxin A and Diphtheria toxin, and plant toxins, for example momordin and saponarin. In addition, certain cytokines such as tumor necrosis factor (TNF) are cytotoxic. It has been found, however, that immunoconjugates of PAP, a toxin obtained form *Phytolacca americana,* cannot be purified by the method of the present invention.

Immunoconjugates, for purposes of the invention, may be defined as an antibody or antibody fragments such as Fab and $F(ab')_2$ that selectively bind to an epi-

tope, covalently bound to a protein toxin. More generally, toxin conjugates may be prepared by the method of the invention. A "toxin conjugate", as used herein, means a protein toxin covalently bound to a selective binding molecule. Such selective binding molecules may include, in addition to antibodies and the selective binding fragments thereof mentioned above, hormones, cytokines such as TNF, lymphokines such as interleukin 1 or 2, and cell growth factors such as transferrin, epidermal growth factor and bombesin. Such selective binding molecules bind to receptors found on the target cells to which these molecules bind. Immunoconjugates also selectively bind to cells; however, such binding is based generally upon affinity and avidity for a particular epitope associated with the target cell to which the immunoglobulin portion of the immunoconjugate binds.

While suitable method of preparing such immunoconjugates are known in the art (see, e.g., Miyazaki et al., supra, Lambert et al., supra, and U.S. Pat. No. 4,340,535 to Voisin et al.), a brief summary of the procedure used by applicants follows.

Monoclonal antibodies (designated in the Examples below as MAB260F9) of the IgG class were provided in a phosphate EDTA (P_iEDTA) solution containing about 0.10M Na_2PO_4 and 1 mM (minimum) EDTA. In order to prepare the antibodies for coupling to the free thiol on the ricin A chain, the Ig was derivatized with DTNB (dithionitrobenzoic acid) and iminothiolane (IT), at about 0° C. for a reaction time of about 24 hours. The Ig-TNBIT complex was then desalted using a Trisacryl GF-05 column (LKB, Bromma, Sweden) buffered to a pH of about 8.0 with P_iEDTA.

Soluble recombinant ricin A (srRTA) was provided by the method described in co-pending application Ser. No. 837,583 for "Recombinant Ricin Toxin Fragments." filed Mar. 7, 1986 and of common assignment herewith. The disclosure of that application is hereby incorporated by reference in its entirety.

The srRTA, at an initial concentration of about 10 mg/ml in P_iEDTA containing 0.1% β-mercaptoethanol (BME), was clarified by centrifugation (~1000 rpm) and desalted on a Trisacryl GF-05 column as above. A free thiol assay was run using DTNB and uv spectroscopy to assay released TND (peak at 412 nm).

The immunoconjugates were then prepared by adding about 10–30 vol. % of glycerol to the srRTA, followed by addition of the Ig-TNB-IT complex. The crude conjugate mixture was allowed to sit at room temperature for about 2 hours, at which time the conjugation process was presumed to be complete.

Purification of the crude conjugate mixture and removal of unconjugated Ig is carried out as follows:

According to the purification method of the present invention, the crude conjugate mixture as prepared above is first loaded onto a sizing column to remove unreacted srRTA and any high molecular weight aggregates. A suitable column for this step is Sephacryl S-300 (Pharmacia, Inc., Piscataway, N.J.), preferably equilibrated prior to use with a phosphate buffer (pH between about 6 and 7). The eluted conjugate mixture, in P_iEDTA, is at this point loaded onto a column preequilibrated in the same solution as the conjugate mixture outlined above, further containing 1 M NaCl, and packed with a relatively strongly hydrophobic gel such as Phenyl Sepharose CL-4B ® (manufactured by Pharmacia) or TSK Phenyl-5PW (Toyo Soda Kogyo K.K.).

With a Phenyl Sepharose column, the buffer used in both the sizing step and the subsequent chromatographic separation step preferably contains sodium chloride. With TSK Phenyl-5PW, ammonium solfate is the preferred alternative. Initial concentration of the salt is preferably about 1 M, the concentration used gradually decreasing with each column volume eluting the conjugate from the hydrophobic gel.

Immunoconjugate and unconjugated Ig are then separated and removed from the column as follows. Between about 4 and 10 column volumes of salt solutions (as above) successively decreasing in salt concentration are used to elute the various species. Optionally, increasing concentrations of an organic solvent such as glycerol, ethanol or propylene glycol may be added to the eluant solution to obtain the conjugate mixture in a more concentrated form. Non-conjugated Ig is eluted first, followed by various "mers" (e.g., first by a "1-mer", an Ig conjugated to one A-chain, followed by a "2-mer", an Ig conjugated to two A-chains, etc., up to a "4-mer").

The immunoconjugate so isolated may then if desired be concentrated, e.g. by ultrafiltration, and desalted on a suitable column such as Trisacryl or Sephadex. The desalted immunoconjugate is filtered through a 0.2μ filter. A preferred final concentration of the purified immunoconjugate for medical use is at least about 4 mg/ml, and recoveries on the order of at least about 50–60% are typically obtained with this procedure.

In an alternative embodiment of the invention, a modified hydrophobic gel is provided for a "fast flow" chromatographic separation and purification step. The gel is either Phenyl Sepharose or TSK Phenyl-5PW, preferably Phenyl Sepharose, modified so as to contain only half the number of phenyl groups normally present. Such a modified gel is less hydrophobic, and thus does not bind the conjugate or Ig quite as strongly. Unconjugated Ig is removed with the first column volume of phosphate buffer/salt solution, as described above, and immunoconjugate is removed, typically, with a second column volume of phosphate buffer containing 10–60 vol. % of an organic solvent. In this procedure, the concentration of sodium chloride or ammonium sulfate in the first column volume of eluant, depending on the modified gel selected as above, is about 1.5M. Immunoconjugate is removed in this manner at a concentration of at least about 4 mg/ml, obviating the necessity for a concentration step following removal from the column.

It is to be understood that while the invention has been described in conjunction with the preferred specific embodiments thereof, that the foregoing description as well as the examples which follow are intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims.

EXAMPLE 1

Monoclonal antibodies (designated MAB260F9) of the IgG class were obtained in P_iEDTA (0.10M $NaPO_4$, 1 mM EDTA, pH 8.0) at a concentration of 33.18 mg/ml. Purification was effected using a DEAE Sepharose (Pharmacia) column and ultrafiltration (0.2μ). The Ig was assayed for free thiols using dithionitrobenzoic acid (DTNB) and uv spectrometry to monitor released TNB groups, and it was determined that no free thiol groups were present in the Ig solution. Derivatization with DTNB and iminothiolane (IT) in preparation for coupling to the free thiol or ricin A was then accomplished by adding 363 μl of 1 mM DTNB and 525 μl 10 mM IT to the initial 8.71 ml of Ig. The reaction

temperature was maintained at about 0° C. and derivatization was allowed to proceed overnight, i.e. for about 24 hours. The derivatized conjugate was then desalted on a Trisacryl GF-05 column (LKB, Bromma, Sweden) buffered to a pH of 8.0 with P_iEDTA and using a flow rate of about 25 ml/hr.

Soluble recombinant ricin A chain (srRTA) was obtained by the method set forth in co-pending U.S. Patent Application Ser. No. 837,583. The srRTA was provided at an initial concentration of 10 mg/ml in P_iEDTA with 0.1% β-mercaptoethanol (BME) added. Contaminating particulate matter was removed by centrifugation of about 1000 rpm and desalting on a Trisacryl GF-05 column using P_iEDTA and a flow rate of about 25 ml/hr. A free thiol assay was run as described above, and it was determined that approximately 0.73 free thiols were present per molecule of srRTA.

Conjugation was accomplished by adding about 5 ml glycerol to the about 21.1 ml of desalted srRTA, followed by about 10.9 ml of Ig-TNB-IT complex prepared above. The reaction was allowed to proceed at about 25° C. for two hours, at which time it was presumed that conjugation was complete. A free thoil assay at this point gave a 96% conjugation efficiency.

The crude conjugate mixture so obtained was loaded onto a 950-ml Sephacryl S-300 column to remove unreacted srRTA and high molecular weight aggregates. The column was preequilibrated with a sodium chloride/phosphate buffer (pH 6.5; 0.1M Na_2PO_4; 1M NaCl; 1 mM EDTA). The conjugate was eluted with the buffer at a flow rate of about 40 ml/hr.

The resulting mixture, containing unconjugated Ig as well as various Ig/srRTA conjugates, was then loaded onto a 70 ml Phenyl Sepharose CL-4B column preequilibrated with the phosphate buffer of the preceding step. The initial conjugate pool was about 262 mg in 126 ml solution. Initial elution of the unreacted Ig was accomplished with a column volume of a 1M NaCl solution at a flow rate of about 20 ml/hr. Various "mers" of the immunoconjugate were then eluted as NaCl solutions of decreasing concentration were applied to the column (gradually decreasing from 1M to 0M), these NaCl solutions also containing increasing amounts of glycerol (gradually increasing from 0 (vol.)% to 60 (vol.)%, beginning with a 1:1 Ig:srRTA conjugate ("1-mer") and ultimately yielding a 1:4 Ig:srRTA conjugate ("4-mer"). The mixture was concentrated by ultrafiltration to about 4 mg/ml, desalted on a Trisacryl column as above, and filtered using a 0.2μ filter. Distribution and purity of the final immunoconjugate preparation was assayed by SDS polyacrylamide gel electrophoresis at: 44.0% 1-mer; 30.8% 2-mer; 10.7% 3-mer; 2.7% 4-mer. Cytoxicity as measured by $TCID_{50}$ (MCF-7 cells): 0.004 nM (minus lactose).

EXAMPLE 2

Immunoconjugate purification using the modified "fast flow" hydrophobic column: MAB260F9 antibodies and srRTA were obtained, purified and conjugated as in Example 1. The conjugate mixture was desalted using a Trisacryl column as described in Example 1, and applied to a hydrophobic gel column as follows.

The hydrophobic gel used in this example was Phenyl Sepharose Cl-4B modified by the manufacturer so as to reduce the standard number of phenyl groups by about 50%. The column (d1 cm; vol.3.14 ml) was equilibrated with 10 column volumes of P_i EDTA solution (0.1M Na_2PO_4, 1 mM EDTA) also containing 1.5M NaCl at a pH of about 8.0. The flow rate was set to about 0.13 ml/min and two eluting solutions were prepared: (A) 100 mM Na_2PO_4, pH 8.0, 1 mM EDTA, 1.5M NaCl; and (B) 100 mM Na_2PO_4, pH 8.0, 60 (vol.) % glycerol. The conjugate mixture was loaded onto the column, and unconjugated Ig was initially removed with solution (A) followed by removal of conjugate with solution (B). The column was then rinsed with 1 column volume of solution (B) to ensure complete removal of immunoconjugate.

EXAMPLE 3

Purification of a TNF Immunoconjugate

Purification of TNF Mutein

E. coli cells containing plasmid pAW731 were grown in a suitable growth medium for *E. coli* and were induced to produce TNF. The *E. coli* strain carrying pAW731 has been described in U.S. patent application Ser. No. 753,717, filed July 10, 1985, assigned to the same assignee as the present invention and incorporated herein by reference. The TNF produced by the strain had a single cysteine residue. After induction, the cells were removed from the medium and frozen. The cells were thawed, suspended in 100 ml 0.1M Tris, pH 8, 1 mM EDTA, and sonicated for 30 minutes.

The sonicated cells were centrifuged for 40 min at 12,000 g. The supernatant was removed, adjusted to 0.1M NaCl, and loaded onto a Phenyl Sepharose column previously equilibrated with 0.1M NaCl. The TNF eluted from the column in the flow through, and was dialysed against 0.1M Tris at pH 8.5, 1 mM EDTA. The dialysis retentate was loaded on a DEAE Sepharose column equilibrated with 0.01 M Tris, pH 8.5, 1 mM EDTA, and the TNF was eluted with 0.1M Tris, pH 8.5. The first protein fraction consisted of 95% pure TNF.

Murine monoclonal antibody 317G5 is described in U.S. patent application Ser. No. 690,750, filed Nov. 11, 1985, assigned to the same assignee as the present invention and herein incorporated by reference. 317G5 was derivatized with SPDP as described in U.S. patent application Ser. No. 690,750. Briefly, N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP) was added in a 20-fold molar excess to antibody. Following a 30 minute incubation at room temperature, the unreacted SPDP was removed by dialysis against PBS.

Conjugation

The SPDP-treated antibody was conjugated with TNF. Immediately prior to conjugation, the TNF was reduced with 50 mM dithiothreitol, then desalted on a column of chromatographic resin containing agarose, dextran and/or acrylamide to remove DTT from the protein. Reduced TNF was added in the three to five fold molar excess to the deriatized antibody, and the reaction was allowed to run overnight at 4° C.

The conjugate was loaded onto a Phenyl Sepharose column equilibrated with 2M NaCl and PBS. Free antibody was eluted off the column at 0.5M NaCl. The conjugate and free TNF were eluted off the column with PBS and 30% propylene glycol. Free TNF was separated from the conjugate by size exclusion chromatography using a S-200 Sepharose column.

We claim:

1. A method of purifying immunotoxin conjugates, comprising the steps of:

providing a conjugation mixture containing immunotoxin conjugate, unconjugated selective binding molecule and unconjugated toxin protein;

removing said unconjugated toxin protein from said mixture by gel filtration chromatography;

adding said mixture devoid of said unconjugated toxin protein to a hydrophobic gel chromatograph; and

removing said unconjugated binding molecule from said immunotoxin conjugate loaded on a hydrophobic gel with an eluting solution comprising an aqueous salt.

2. The method of claim **1**, wherein said step of removing unconjugated toxin on a sizing column precedes said step of removing the unconjugated binding molecule from toxin conjugate.

3. The method of claim **1**, wherein said step of removing the unconjugated binding molecule from the immunotoxin conjugate precedes the step of removing unconjugated toxin on a sizing column.

4. The method of claim **2**, wherein said toxin protein is a ribosome inactivating protein.

5. The method of claim **4**, wherein said toxin protein is ricin toxin A chain.

6. The method of claim **5**, wherein said ricin toxin A chain is recombinantly produced.

7. The method of claim **1**, wherein said toxin protein is tumor necrosis factor.

8. The method of claim **1**, wherein said binding molecule is selected from the group consisting of antibodies and fragments thereof that selectively bind to an epitope, hormones, cytokines, lymphokines and cell growth factors.

9. The method of claim **8**, wherein said binding molecule is selected from the group consisting of antibodies and fragments thereof that selectively bind to an epitope.

10. The method of claim **1**, wherein said eluting solution comprising an aqueous salt solution contains sodium chloride at a concentration of about 1.0M or less.

11. The method of claim **10**, wherein said aqueous salt solution is buffered to a pH ranging from about 6 to about 8.

12. The method of claim **1**, wherein said aqueous salt solution is in the range of about four and ten column volumes, each successively decreasing in salt concentration to about 0.5M.

13. The method of claim **1**, wherein said salt solution further comprises an organic solvent.

14. The method of claim **12**, wherein said column volumes of salt solution include an organic solvent increasing in amount up to about 60 volume percent.

15. The method of claim **14**, wherein said organic solvent is selected from the group consisting of glycerol, propylene glycol and ethanol.

16. A method of purifying immunotoxin conjugates, comprising the steps of:

providing a conjugation mixture containing immunoconjugate, unconjugated Ig and unconjugated ricin A chain;

removing said unconjugated ricin A chain from said conjugation mixture by gel filtration chromatography;

loading said mixture devoid of said unconjugated ricin A onto a colum filled with hydrophobic gel; and

removing said unconjugated Ig with at least one column volume of an aqueous salt solution.

17. The method of claim **16**, wherein said gel is provided with a number of phenyl groups at least sufficient to ensure hydrophobic retention of said conjugate mixture.

* * * * *

Exhibit 3

United States Patent [19]

Scannon et al.

[11] **Patent Number: 4,590,071**

[45] **Date of Patent: May 20, 1986**

[54] **HUMAN MELANOMA SPECIFIC IMMUNOTOXINS**

[75] Inventors: **Patrick J. Scannon**, Davis; **Lynn E. Spitler**, Tiburon; **Howard M. Lee**; **Russell T. Kawahata**, both of San Francisco; **Ronald P. Mischak**, Palo Alto, all of Calif.

[73] Assignee: **Xoma Corporation**, Berkeley, Calif.

[21] Appl. No.: **654,613**

[22] Filed: **Sep. 25, 1984**

[51] **Int. Cl.**[4] **A61K 39/395**; A61K 39/44; C07K 17/06

[52] **U.S. Cl.** **424/85**; 530/391; 530/413; 530/387

[58] **Field of Search** ..... 260/112 R, 112 B; 424/85

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Primary Examiner—Howard E. Schain

Attorney, Agent, or Firm—Townsend and Townsend

[57] **ABSTRACT**

Conjugates of monoclonal antibodies specific to human melanoma and the A chain of a toxic lectin such as ricin or an equivalent ribosomal inhibiting protein. The conjugate is synthesized by a novel process employing anti-toxic lectin B chain antibodies to remove lectin B chain impurities and provide a highly purified conjugate that is non-toxic to cells other than melanoma. The conjugates are used to treat human melanoma.

The hybridomas XMMME-001 and XMMME-002 were deposited with the American Type Culture Collection (A.T.C.C.) on Mar. 26, 1985, and given A.T.C.C. Accession Nos. HB8759 and HB8760, respectively.

20 Claims, 8 Drawing Figures

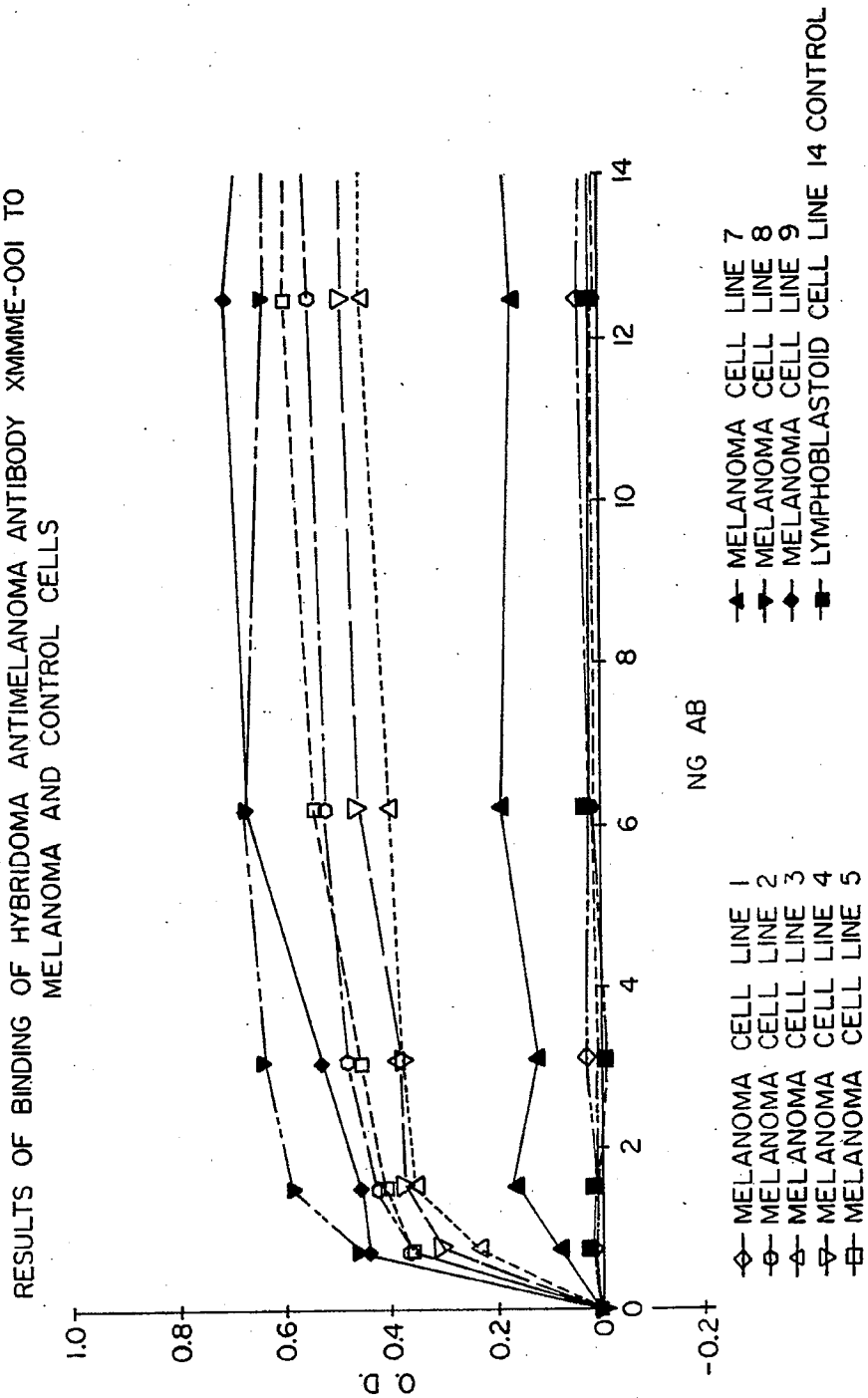
RESULTS OF BINDING OF HYBRIDOMA ANTIMELANOMA ANTIBODY XMMME-001 TO MELANOMA AND CONTROL CELLS
O.D.
1.0
0.8
0.6
0.4
0.2
0
-0.2
0
2
4
6
8
10
12
14
NG AB
MELANOMA CELL LINE 1
MELANOMA CELL LINE 2
MELANOMA CELL LINE 3
MELANOMA CELL LINE 4
MELANOMA CELL LINE 5
MELANOMA CELL LINE 6
MELANOMA CELL LINE 7
MELANOMA CELL LINE 8
MELANOMA CELL LINE 9
LYMPHOBLASTOID CELL LINE 14 CONTROL

FIG. 1.

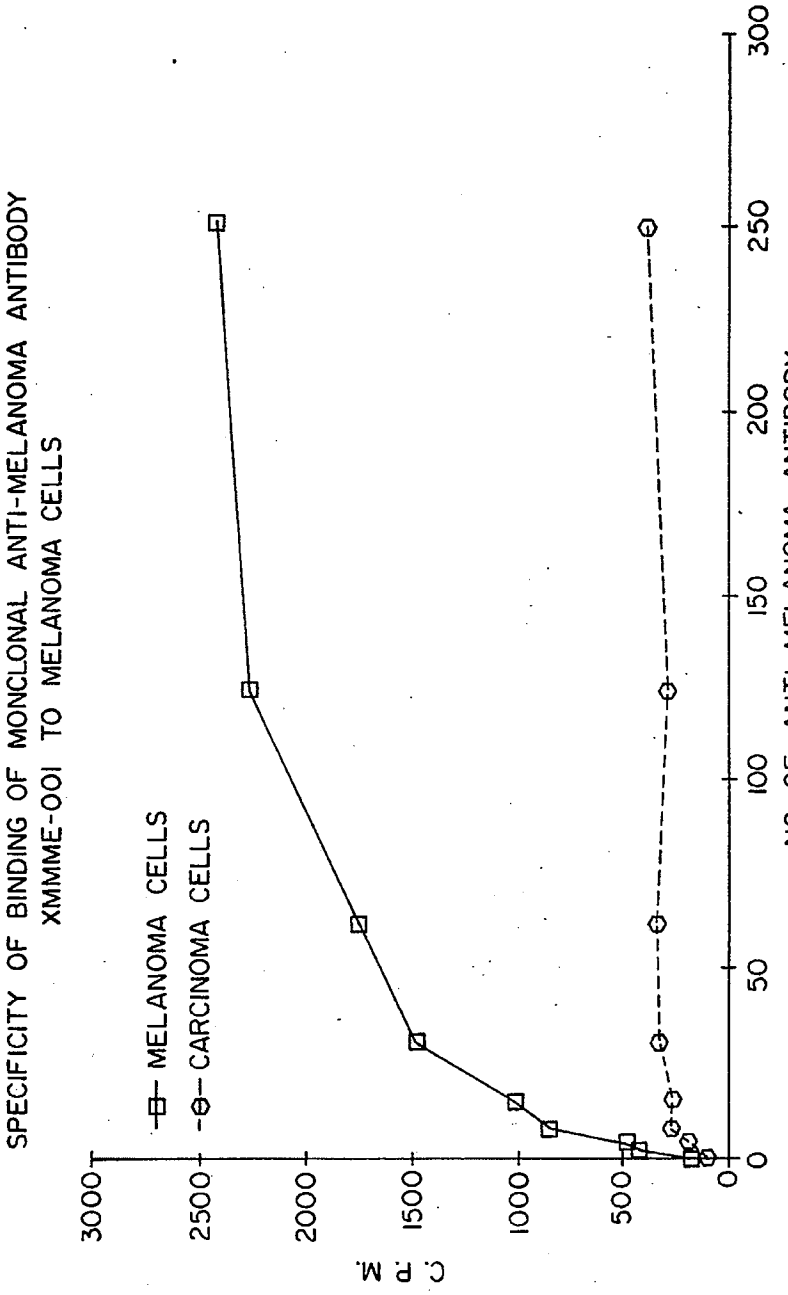
SPECIFICITY OF BINDING OF MONCLONAL ANTI-MELANOMA ANTIBODY XMMME-001 TO MELANOMA CELLS
MELANOMA CELLS
CARCINOMA CELLS
C. P. M.
3000
2500
2000
1500
1000
500
0
0
50
100
150
200
250
300
NG OF ANTI-MELANOMA ANTIBODY

FIG. 2.

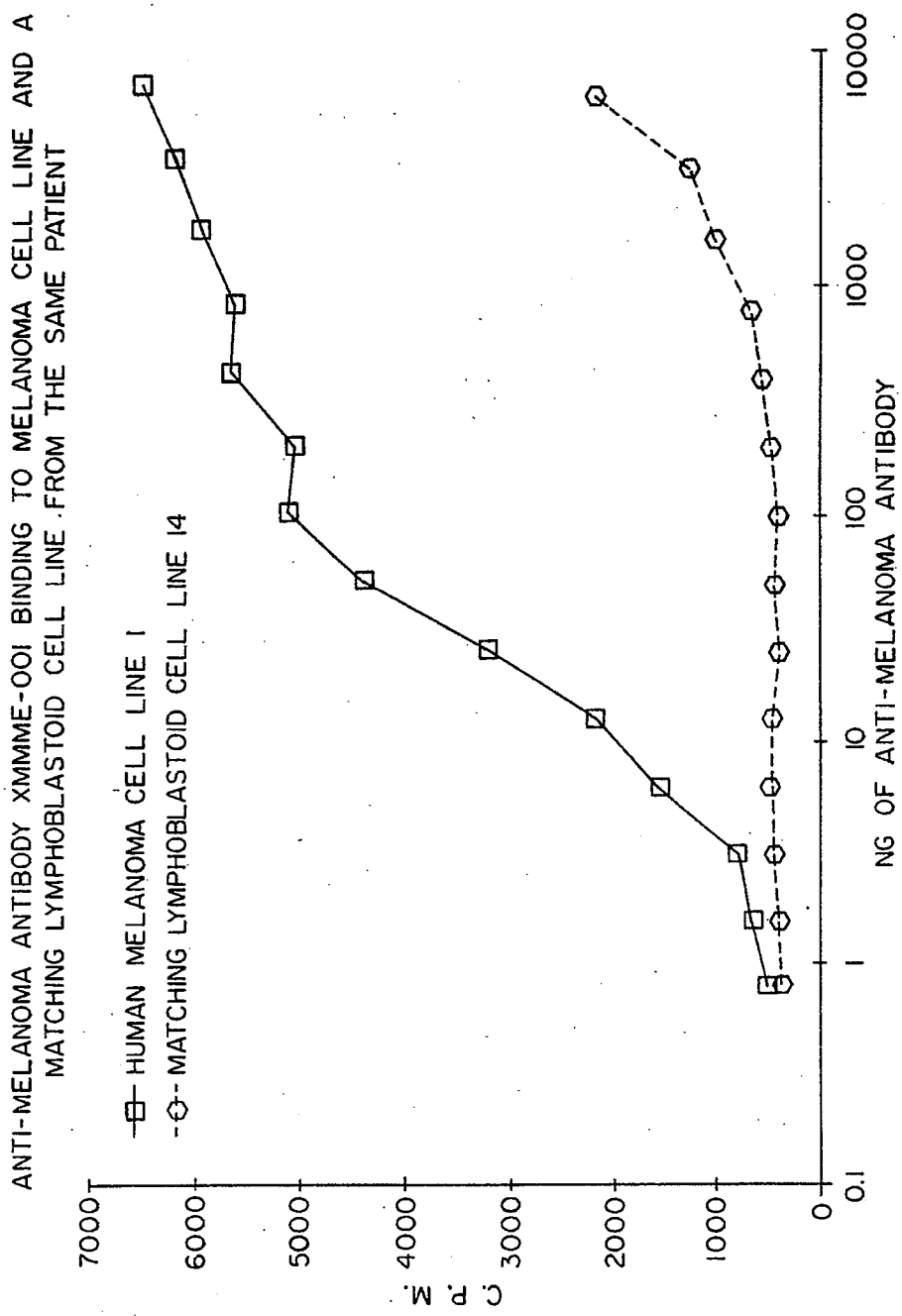
ANTI-MELANOMA ANTIBODY XMMME-001 BINDING TO MELANOMA CELL LINE AND A MATCHING LYMPHOBLASTOID CELL LINE FROM THE SAME PATIENT
HUMAN MELANOMA CELL LINE 1
MATCHING LYMPHOBLASTOID CELL LINE 14
C.P.M.
7000
6000
5000
4000
3000
2000
1000
0
0.1
1
10
100
1000
10000
NG OF ANTI-MELANOMA ANTIBODY

FIG. 3.

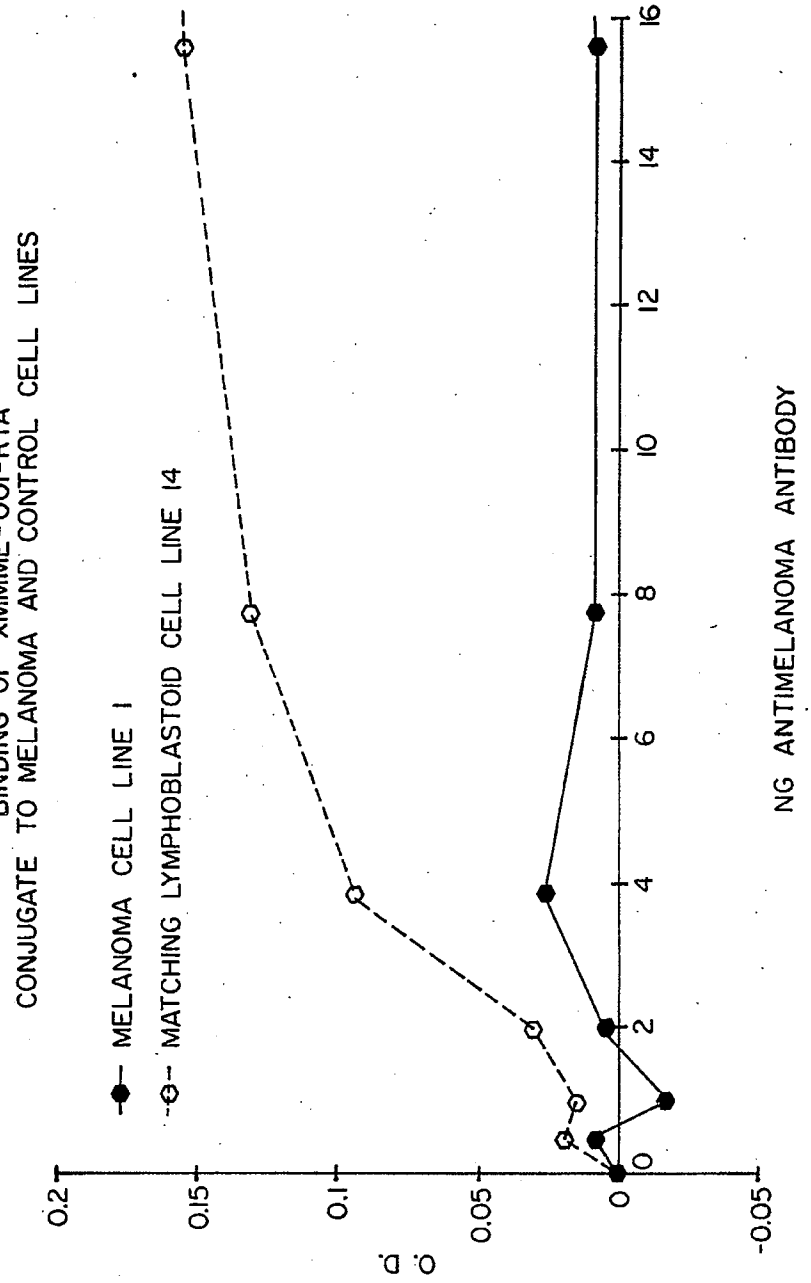
BINDING OF XMMME-001-RTA
CONJUGATE TO MELANOMA AND CONTROL CELL LINES
MELANOMA CELL LINE 1
MATCHING LYMPHOBLASTOID CELL LINE 14
O. D.
0.2
0.15
0.1
0.05
0
-0.05
0
2
4
6
8
10
12
14
16
NG ANTIMELANOMA ANTIBODY

FIG._4.

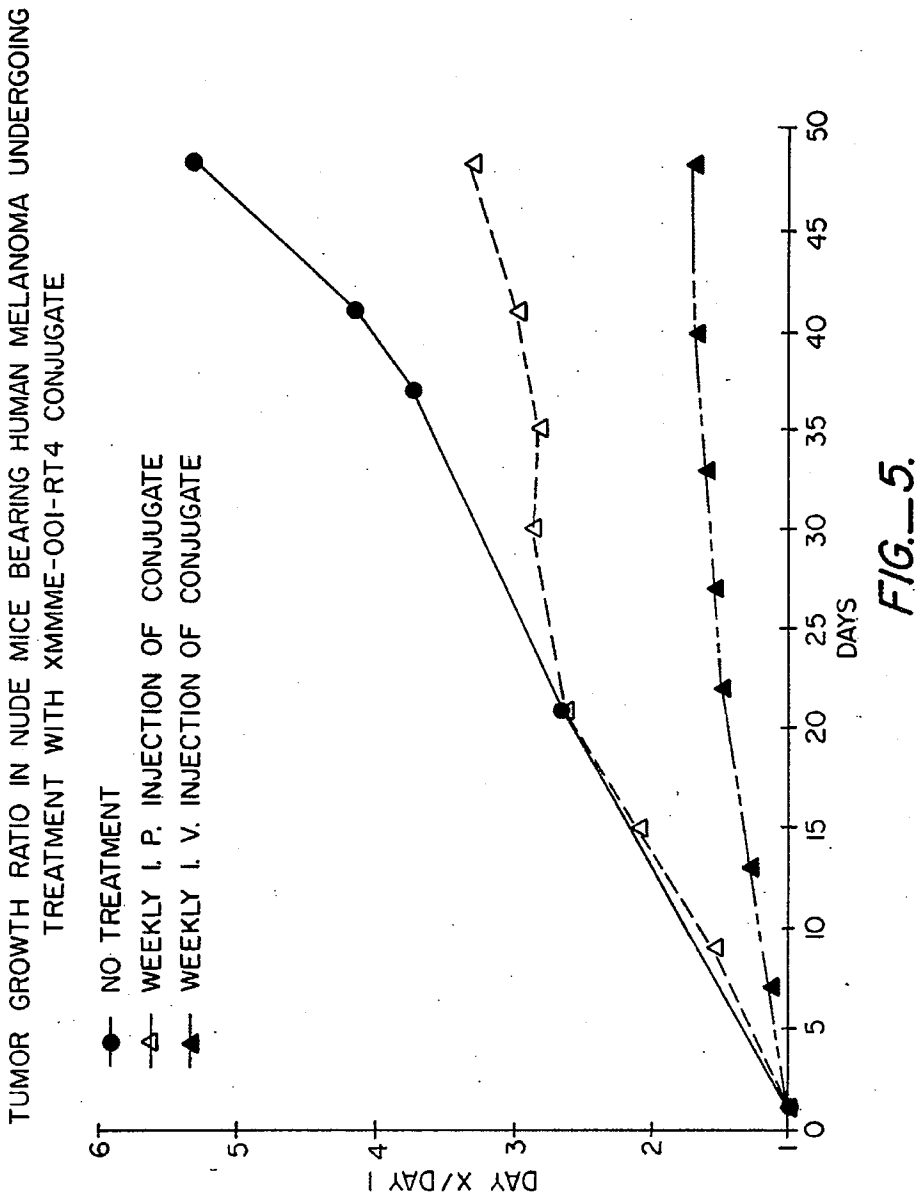
TUMOR GROWTH RATIO IN NUDE MICE BEARING HUMAN MELANOMA UNDERGOING TREATMENT WITH XMMME-001-RT4 CONJUGATE
NO TREATMENT
WEEKLY I. P. INJECTION OF CONJUGATE
WEEKLY I. V. INJECTION OF CONJUGATE
DAY X/DAY 1
DAYS
0
5
10
15
20
25
30
35
40
45
50
1
2
3
4
5
6

FIG._5.

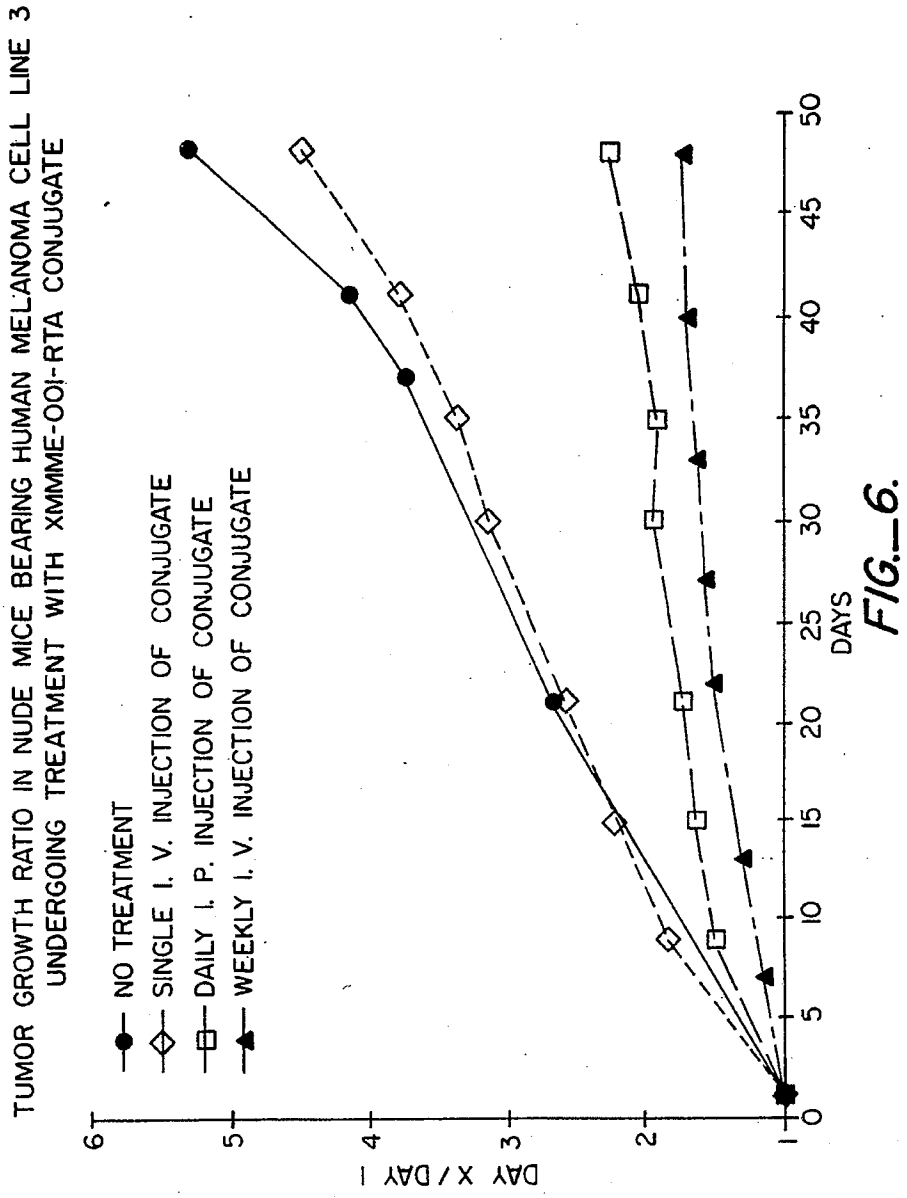
TUMOR GROWTH RATIO IN NUDE MICE BEARING HUMAN MELANOMA CELL LINE 3
UNDERGOING TREATMENT WITH XMMME-OOI-RTA CONJUGATE
NO TREATMENT
SINGLE I. V. INJECTION OF CONJUGATE
DAILY I. P. INJECTION OF CONJUGATE
WEEKLY I. V. INJECTION OF CONJUGATE
DAY X / DAY 1
DAYS
0
5
10
15
20
25
30
35
40
45
50
1
2
3
4
5
6

FIG._6.

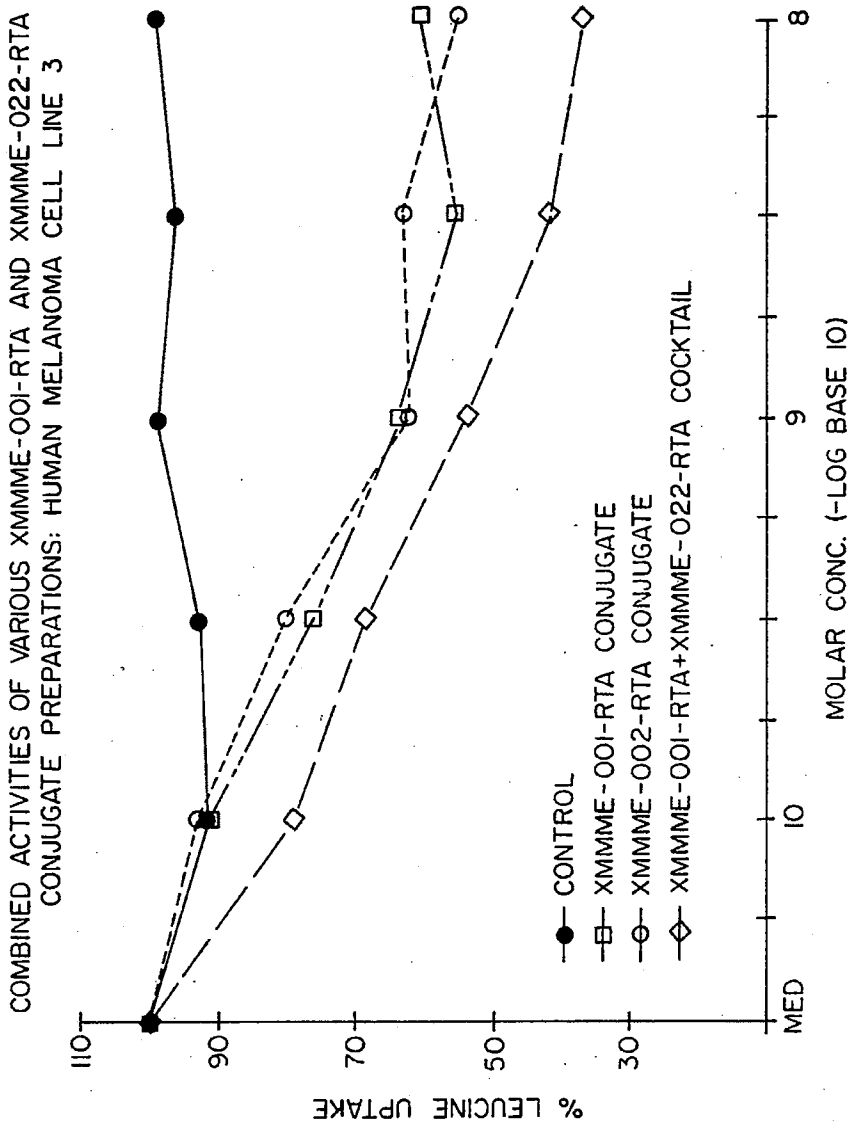
COMBINED ACTIVITIES OF VARIOUS XMMME-001-RTA AND XMMME-022-RTA CONJUGATE PREPARATIONS: HUMAN MELANOMA CELL LINE 3
% LEUCINE UPTAKE
110
90
70
50
30
MED
10
9
8
MOLAR CONC. (-LOG BASE 10)
CONTROL
XMMME-001-RTA CONJUGATE
XMMME-002-RTA CONJUGATE
XMMME-001-RTA+XMMME-022-RTA COCKTAIL

FIG. 7.

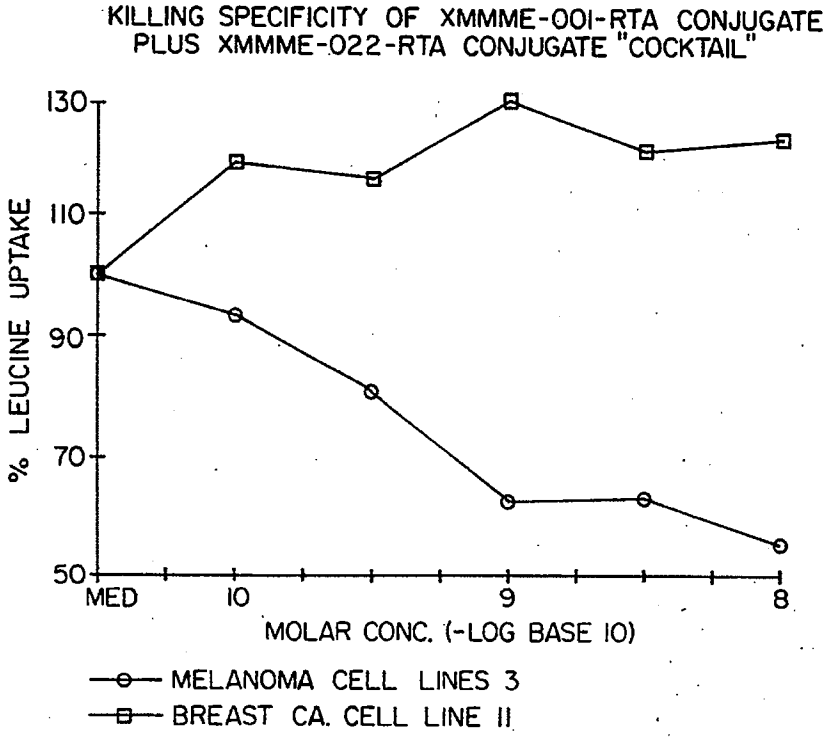
KILLING SPECIFICITY OF XMMME-001-RTA CONJUGATE
PLUS XMMME-022-RTA CONJUGATE "COCKTAIL"
% LEUCINE UPTAKE
130
110
90
70
50
MED
10
9
8
MOLAR CONC. (-LOG BASE 10)
MELANOMA CELL LINES 3
BREAST CA. CELL LINE 11

FIG. 8.

HUMAN MELANOMA SPECIFIC IMMUNOTOXINS

This invention relates to cytotoxic products for the treatment of cancer and, specifically, to cytotoxic products formed by binding the A chain of a toxic lectin or its equivalent, such as the class of materials known as ribosomal inhibiting proteins (RIP), to a human melanoma specific monoclonal antibody; methods for production of such products; and, methods for the use of such products in the treatment of human melanoma.

BACKGROUND OF THE INVENTION

The use of cytotoxic products in the treatment of cancer is well known. Equally well known are the difficulties associated with such treatment. Of these difficulties, the lack of cancer-specific cytotoxicity has received considerable attention, albeit with marginal success. Cytotoxic products continue to kill cancer cells and normal cells alike. Such non-specificity results in a number of undesirable side effects for patients undergoing cancer chemotherapy with cytotoxic products including nausea, vomiting, diarrhea, hemorrhagic gastroenteritis, and hepatic and renal damage. Due to normal cell toxicity, the therapeutic dosage of cytotoxic products has been limited such that cancerous cells are not killed to a sufficient level that subsequently prevents or delays new cancerous growth.

The cytotoxic action of toxic lectins, and especially that of ricin and abrin, has been well studied. It is known that toxic lectins consist of two polypeptide chains, A and B, linked by means of disulfide bridge(s). Cytotoxicity is associated with the A chain and its inhibition of protein synthesis in nucleated cells. The B chain is essentially a delivery vehicle for the A chain. The B chain recognizes polysaccharide units at the surface of cells and creates a high affinity interaction with such units. Once the B chain binds with polysaccharide units at the cell surface, the A chain is incorporated into the cell, block ribosomal protein synthesis and untimately leading to cell death.

Toxic lectins of the type of structure and function similar to ricin include abrin, modeccin and mistletoe toxin. One other category of ribosomal inhibiting protein (RIP) is the toxin with only one subunit having functional characteristics analogous to ricin A chain. This type of RIP lacks cytotoxicity to the intact cell because of the inherent absence of a binding fragment analogous to ricin B chain. Examples of RIP's of this latter type include gelonin and pokeweed antiviral protein.

SUMMARY OF THE INVENTION

The present invention overcomes the difficulty of non-specific cytotoxicity in human melanoma chenotherapy by chemically bonding the A chain of a toxic lectin, such as ricin or abrin, with monoclonal antibodies specific to human melanoma (MoAbHM) to form cytotoxic products called conjugates using the naturally existing sulfhydryl group to form a disulfide bridge. In the conjugates, the MoAbHM assumes the role of the B chain in whole lectins. That is, the MoAbHM functions as the delivery vehicle for the toxic A chain, delivering the toxin specifically to human melanoma cells. Specificity is achieved through the selective binding activity of the monoclonal antibody with epitopes of human melanoma associated antigens. The bonding of the toxic lectin A chain to the monoclonal antibody blocks the toxic effect of the A chain until the monoclonal antibody has complexed with the human melanoma cell. The toxin is then incorporated into the cell at which point it blocks protein synthesis and the melanoma cell dies.

In a presently preferred embodiment of the invention, the conjugate comprises the XMMME-001 variety of monoclonal antibodies and the toxic A chain of the lectin ricin. Alternate embodiments of the invention include conjugates utilizing the XMMME-002 variety of monoclonal antibody, which is produced by the same method as the XMMME-001 antibody but recognizes a different epitope on the same melanoma associated antigen, and the toxic A chain of ricin.

According to the invention, any toxic lectin which may be split into A and B polypeptide chains, specifically abrin, may be used in the same way ricin is used in the preferred embodiment. In addition, any RIP, specifically gelonin and pokeweed antiviral protein, may be used in the same way as ricin A chain. Such materials are equivalent to the toxic lectin A chain for purposes of this invention.

Experiments using the invention have verified both the specificity of these cytotoxic products toward human melanoma cells and the killing of such cells.

The invention also contemplates the use of different conjugates in a therapeutic cocktail wherein a first conjugate and at least a second conjugate are administered either as a mixture, individually in a set sequence or in combination with at least one other cancer therapeutic agent.

Cytotoxic products using the A chain of a toxic lectin are known in the prior art. ["Selective Killing of Normal or Neoplastic B Cells by Antibodies Coupled to the A Chain of Ricin", K. A. Krolick, et al., *Proc. Natl. Acad. Sci. USA, Vol.* 77, No. 9, pp. 5419–5423, September 1980; "Monoclonal Antibody-Toxin Conjugates: Aiming the Magic Bullet", Philip E. Thorpe, et al., *Monoclonal Antibodies in Clinical Medicine,* Academic Press, pp. 168–190 (1982); "Cytotoxicity Acquired by Conjugation of an Anti-$Thy_{1.1}$ Monoclonal Antibody and the Ribosome-Inactivating Protein, Gelonin", Philip E. Thorpe, et al., *Eur. J. Biochem.,* 116 447–454 (1981); "Antibody-Directed Cytotoxic Agents: Use of Monoclonal Antibody to Direct the Action of Toxin A Chains to Colorectal Carcinoma Cells", D. Gary Gilliland, et al., *Proc. Natl. Acad. Sci. USA,* Vol. 77, No. 8, pp. 4539–4543, August 1980.] The present invention is an improvement over the prior art in that the purification of the A chain yields a pure product free of intact toxin molecules. This reduced toxicity permits administration of larger, more efficacious doses of the final conjugates.

According to the invention, this improved purity is achieved by running a quantity of toxic lectin A chain split from whole toxic lectin through a chromatography column containing a support media comprising anti-toxic lectin B chain antibodies and modified gels. This step removes any toxic lectin B chain impurities, thus reducing recombination of the A and B chains into the more toxic whole lectin.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

Materials And Methods

1. Ricin Extraction from Whole Castor Beans

Ricin is extracted from the castor bean (*Ricinus communis*) by known methods. Essentially, whole castor beans are homogenized in the presence of phosphate buffered saline (PBS) with azide pH 7.2 (PBS with Azide, pH 7.0: 10mM sodium phosphate, 0.15M NaCl, 0.02% NaN_3, 0.5% phenylmethanesulfonyl fluoride (PMSF), pH 7.0.) The homogenate is centrifuged and the supernatant removed by vacuum aspiration leaving a bean pellet and fat lipid layer. The castor bean supernatant is precipitated with 60% ammonium sulfate (A.S.) with stirring and refrigeration. The 60% A.S. precipitate is collected by centrifugation, then dissolved in a minimum of PBS with azide, pH 7.2. This solution is then dialyzed against PBS with azide, pH 7.2 until A.S. free.

The castor bean supernatant is applied to a BioGel A-15 column. Since the ricin has a moderate affinity to the carbohydrate moieties of the column resin, this results in a delay of the elution of the toxin from the column and the separation from the run-through fraction which consists of lipids, pigments, unwanted proteins and other substances. Addition of 50 mM N-acetylgalactosamine in PBS with azide, pH 7.2 reverses the affinity of the ricin and results in a sharp peak corresponding to the elution of the bound ricin toxin.

2. Ricin Toxin A Chain Separation

The ricin toxin A chain (RTA) is separated from whole ricin obtained above by Affinity Chromatograph (AC) using an acid treated Sepharose column. (Sepharose is the exclusive trademark of Pharmacia Fine Chemicals.) Sepharose is a bead-formed gel prepared from agarose. In its natural state agarose occurs as part of the complex mixture of charged and neutral polysaccharides referred to as agar. The agarose used to make Sepharose is obtained by a purification process which removes the charged polysaccharides to give a gel with only a very small number of residual charged groups.

The column is first equilibrated with PBS with azide, pH 6.5. The ricin sample is then applied to the column and washed with PBS with azide and the eluate of non-binding proteins discarded. The column is then washed with a reducing buffer: (0.5M TRIS-HCl, pH 7.7, 1M β-mercaptoethanol, 1.2 mM EDTA.) The fraction containing the RTA is collected and dialyzed against PBS with azide, pH 6.5 until the reducing buffer has been removed. The dialyzed RTA is then filtered through glass fiber filter paper and subjected to AC again using an acid treated Sepharose column. The flow-through non-binding protein peak contains RTA and is collected. The column is then washed with PBS with azide, pH 6.5 until the entire RTA peak has been collected.

3. Ricin Toxin A Chain Purification

The RTA obtained above is purified to remove ricin toxin B chain (RTB) impurities. This step is essential to prevent increased toxicity to non-melanoma cells due to residual whole ricin toxin following separation.

The RTB is removed by first filtering the RTA fraction collected above through a glass fiber filter paper and then applying RTA to a Sepharose column previously coupled with goat anti-RTB antibodies. The flow through protein peak of RTA is collected by washing with PBS with azide, pH 6.5. After the sample is dialyzed and concentrated, the RTA is added to an equal volume of cold 100% glycerol and adjusted to 10^{-5}M mercaptoethanolamine and stored at −20° C.

Quality control tests are performed on the purified RTA. Discontinuous SDS-PAGE on 12.5% gel indicates an absence of any contaminating band corresponding to native ricin and elicits only line bands at 33 kilodaltons (kD) and 30 kD associated with RTA isomers. IEF on LKB Ampholine PAG-plates or on Serva Servalyt Precotes failed to reveal any bands corresponding to either native recin or ricin B chain and only Coomassie Blue stained bands have appeared corresponding to RTA.

4. Human Melanoma Specific Monoclonal Antibody Production

In a presently preferred embodiment of the invention, the MoAbHM used is of the IgG2a subclass produced according to known hybridization procedures described by Kohler and Milstein, *Eur. J. Immunol.*, 6:292 (1976) with minor modifications by Hocibe, et al., described in Human Leucocyte Markers by Monoclonal Antibodies, Springer-Verlag, Berlin (in press). Cultured M21 human melanoma cells were used as the immunogen for all MoAbHM varieties including the XMMME-001 antibody produced by XMMME-001 hybridomas used in the preferred embodiment. In an alternate embodiment of the invention, the related XMMME-002 antibody is produced by the same methods. These antibodies differ in that they react with different epitopes of the same melanoma associated antigen of approximately 240 kD/>480 kD M.W.

The hybridomas XMMME-001 and XMMME-002 were deposited with the American Type Culture Collection (A.T.C.C.) on Mar. 26, 1985, and given A.T.C.C. Accession Nos. HB8759 and HB8760, respectively.

5. Reduction of RTA

RTA is reduced with dithiothreital (DTT) prior to its reaction with XMMME-001 antibody to form XMMME-001-RTA conjugates. This reduces the SH group on the RTA molecule, facilitating the formation of disulfide bridges with XMMME-001 antibody and the subsequent formation of the conjugates.

1M DTT, pH 7.0, is added to room temperature RTA solution to a final concentration of 50 mM DTT and incubated at 4° for 8–12 hours. This solution is applied to a Gel Permeation Chromatography (GPC) desalting column pre-equilibrated with phosphate buffered saline (PBS) with azide, pH 7.0 and washed with the same buffer. The first peak, which elutes in the void volume, contains RTA-SH and is collected. The RTA-SH is concentrated to a desired concentration, filtered and used in the conjugation reaction described below.

6. Thiol Group Addition to MoAbHM

Prior to the conjugation reaction, XMMME-001 antibody is activated with N-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) (312 M.W.) which facilitates the formation of disulfide bridges with RTA-SH in the conjugation reaction.

An amount of XMMME-001 antibody (150,000 M.W., approximate), 10–20% more than the amount of conjugate desired, is diluted to a desired concentration with SPDP coupling buffer (0.1M NaP, 0.1M NaCl, 0.02% NaN_3, 0.5% PMSF), pH 7.5. If the final pH is not in the range pH 7.4–7.6, the solution is dialyzed for 8–12 hours at 0°–6° C. against a 5–50 fold or greater volume of SPDP coupling buffer. A 15X molar excess of SPDP (in absolute ethanol) is added dropwise to the XMMME-001 coupling buffer solution with vigorous stirring at room temperature.

The solution is stirred at room temperature such that the SPDP solution is immediately dispersed but not so vigorous that bubbles or frothing are generated. This

solution, containing PDP-XMMME-001, is dialyzed at 0°–6° C. for 16–36 hours against PBS with azide, pH 7.0. Any visible precipitation present in the solution is removed by filtration.

7. Conjugation of XMMME-001 With RTA

The conjugation of PDP-XMMME-001 with RTA-SH is achieved by adding a 3-20X molar excess of RTA to PDP-XMMME-001 with gentle stirring. The mixture is incubated at 2°–6° C., without stirring, for 16–72 hours. The mixture is filtered if a precipitate is seen.

The conjugate of XMMME-001-RTA is purified by GPC. The mixture, containing the conjugate, XMMME-001 antibody and RTA is applied to a column pre-equilibrated with PBS with azide, pH 7.0. The column is run with upward flow and washed with PBS with azide, pH 7.0 until two peaks are eluted. The first peak, which elutes in the void volume, contains the conjugate and XMMME-001 antibody. The second peak contains RTA. The conjugate/antibody mixture is concentrated and dialyzed against PBS without azide, pH 7.0, at 1°–6° C. to remove the azide. The concentration of conjugate is calculated with the aid of a spectrophotometer. SDS-PAGE is performed on a sample of the final product as a quality control measure. The final product is sterilized by ultrafiltration and stored until needed for experimental or clinical use.

According to the invention, the dose of conjugate administered to human patients ranges from 0.01 to 20.0 milligrams of conjugate per kilogram of melanoma cell host body weight (mg/Kg) given at any one time, depending on the therapy protocol. Dilution of the respective conjugates to the desired dosage is achieved with normal saline, other isotonic solution or other injectable adjuvants.

8. Conjugate-Combination Cocktails

The use of chemotherapeutic cocktails for the treatment of cancer is well known. Different chemotherapeutic agents are often used in combination with each other and/or radiation therapy. Administration of these cocktails may be in the form of, for example, a mixture of different agents, a sequence of different agents or a combination of agents administered concurrently with radiation therapy.

According to the invention, human melanoma specific immunotoxins may be administered as therapeutic cocktails as well as separately. In one embodiment of the invention, XMMME-001-RTA conjugate is mixed with an approximately equal amount of XMMME-002-RTA conjugate, produced by the same methods as the XMMME-001-RTA conjugate, to form a cocktail for human melanoma treatment. The dose of any one conjugate, as described above, is reduced by the percentage it represents in the cocktail. For example, if XMMME-001-RTA makes up 50% of the cocktail, the dose of XMMME-001-RTA will range from 0.005 mg/Kg to 10.0 mg/Kg. This cocktail, or any other, may be delivered to the patient by a variety of clinical techniques but generally delivery will be by intravenous infusion over a period of time.

Experimental Results

A variety of in vitro and in vivo experiments utilizing the present invention in one or more embodiments confirm both specificity and cytotoxicity with respect to human melonoma cancer cells. Experimental results indicate a high binding activity on the part of the invention for a variety of human melanoma cell lines. Further, results demonstrate marked blocking of protein synthesis in melanoma cells versus normal cells.

1. In Vitro Results

As discussed above, binding specificity is achieved by replacing the lectin toxin A chain with a human melanoma specific monoclonal antibody. The antibody functions as a delivery vehicle for lectin toxin A chain. As such, the specificity of a conjugate is dependent upon the specificity of its constituent monoclonal antibody toward human melanoma cells.

A number of in vitro studies were conducted for the purpose of ascertaining the binding specificity of the XMMME-001 antibody toward a number of different human melanoma cell lines. Table 1 details the cell lines used in the various studies discussed below. The table lists the cell line code number (also referred to in the figures), the name used by the supplier, the supplier code number, the cell type and the cell lines reaction with the XMMME-001 antibody where a + indicates specificity of the antibody for the particular cell line and a — indicates no appreciable binding activity. Note that Table 1 indicates XMMME-001 specificity toward 8 of 10 human melanomas and non-specificity toward a variety of normal cells as well as several non-melanoma cancers. The binding specificity of the antibody was determined by enzyme-linked immunosorbent assay (EIA) and radioimmunoassay (RIA).

TABLE 1

SOURCES OF CELL LINES USED FOR CHARACTERIZATION OF ANTIMELANOMA ANTIBODY XMMME-001

Code No.	Name used by Supplier	Source	Cell Type of Line	Reaction with XMMME-001
1.	Langenais B	1	Melanoma	+
2.	Carlough B	1	Melanoma	+
3.	Minor	1	Melanoma	+
4.	SH#3	1	Melanoma	+
5.	Gilliam B	1	Melanoma	—
6.	HS 294T	2	Melanoma	+
7.	HS 852T	2	Melanoma	—
8.	HS 936T C	2	Melanoma	+
9.	HS 695 T	2	Melanoma	+
10.	A 375	2	Melanoma	+
11.	MCF-7	3	Breast Ca.	—
12.	HT 29	3	Colon Ca.	—
13.	TE 85	4	Osteogenic Sarcoma	—
14.	Langenais LCL	1	Lympho-blastoid	—
15.	Gilliam LCL	1	Lympho-blastoid	—
16.	PH 342	3	Fibroblast	—
17.	PH 343	3	Fibroblast	—
18.	PH 346	3	Fibroblast	—

1. Dr. B. C. Giovanella, 918 Chenevert Street, Houston, TX 77003
2. Navy Biological Research Laboratory, Naval Supply Center, Oakland, CA
3. Peralta Cancer Research Institute, 3023 Summit, Oakland, CA 94609
4. American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852

FIG. 1 summarizes the results obtained by EIA studies of the binding specificity of the XMMME-001 antibody toward various human melanoma cell lines and normal lymphoblastoid cells. The ordinate represents antibody dose, the abscissa optical density. FIG. 1 shows a marked specificity of the antibody toward melanoma cell lines 1, 3, 4, 6, 8, 9. The lower level of binding seen for lines 2, 5, 7 reflect the lower level or absence of the antigen on these particular cell lines. This is most likely due to loss of antigen from the cell line due

to serial tissue culture passage. The control lymphoblastoid cell line which should not have any antigen present shows on binding of the melanoma antibody XMMME-001.

Studies of the binding specificity of the XMMME-001 antibody toward non-melanoma cancers were also conducted. FIG. 2 illustrates the results of these studies utilizing RIA techniques. Again the ordinate depicts antibody dose but the abscissa shows counts per minute as obtained by using radiolabelled antibody. As may be seen in the figure, XMMME-001 antibody demonstrates little specificity toward non-melanoma carcinoma cells.

RIA techniques were also used to assay studies of the specificity of the antibody with respect to melanoma and lymphoblastoid cells from the same host. The results, summarized in FIG. 3, show a high degree of specificity for the melanoma cells and little binding activity with the lymphoblastoid cells until high doses of antibody are present. Same-host studies were repeated with a conjugate of the XMMME-001 antibody and RTA. These studies, summarized in FIG. 4, indicate specificity of the XMMME-001 RTA conjugate toward the melanoma cells with little binding activity with the matching lymphoblastoid cell line.

The fact that the antibody does not react with lymphoblastoid cells from the same human host as the melanoma cells demonstrates reactivity of the antibody with melanoma associated antigens as opposed to human leukocyte antigen (HLA). Reactivity with HLA has been a problem associated with monoclonal antibodies in the prior art.

2. In Vivo Results

A number of studies using MoAbHM and conjugates of MoAbHM and RTA have been performed on athymic (nude) mice bearing human melanoma tumors. These have been tumor growth studies wherein tumors were measured and surface area calculated periodically over 35 or more days post-treatment. Table II illustrates the results obtained from a number of studies conducted to determine the efficacy of several therapy regimes using the XMMME-001-RTA conjugate. The table details the treatment regime, the administration route (interperitoneal or i.p., and intravenous or i.v.), dose of conjugate (in micrograms), the number of animals used in the particular study, the ratio on day 35 (the area of the tumor on day 35/the area of the tumor on the day treatment started) and the p Value. As can be seen from the table, the greatest efficacy was demonstrated by the administration of the conjugate i.v. on a weekly basis to a total dose of 100 μg per week at the rate of 20 μg per day for five days per week.

TABLE II

RESULTS OF THERAPY WITH ANTIMELANOMA ANTIBODY RICIN A CHAIN CONJUGATES IN NUDE MICE BEARING HUMAN MELANOMAS

TREATMENT	ROUTE	DOSE OF CONJUGATE	NO. OF ANIMALS	RATIO ON DAY 35*
NONE	—	—	20	3.7 ± 0.4
CONJUGATE, SINGLE INJECTION	iv	250 μg	4	3.4 ± 0.3(.686)+
CONJUGATE, WEEKLY INJECTIONS	ip	100 μg	8	2.8 ± 0.2(.157)
CONJUGATE, WEEKLY INJECTIONS	iv	100 μg	6	1.6 ± 0.2(.006)
CONJUGATE, DAILY INJECTION	ip	100 μg	6	1.9 ± 0.4(.018)

*Ratio: Area of tumor on day 35/area of tumor on day treatment started.
+Numbers in parentheses represent p values, two tailed.

FIGS. 5 and 6 graphically illustrate further efficacy studies with nude mice bearing human melanoma tumors. FIG. 5 summarizes the results of studies comparing weekly i.p. versus weekly i.v. injection of XMMME-001-RTA conjugate. Note that weekly i.v. administration substantially reduced tumor growth. FIG. 6 summarizes the results of studies comparing single i.v. injection of conjugate with weekly i.p. and weekly i.v. injection. Again, the weekly i.v. injection (100 μg per week) showed the greatest efficacy with respect to suppressing tumor growth.

3. In Vitro Cocktail Results

The combined activities of the XMMME-001-RTA and XMMME-002-RTA conjugates were studied in vitro using human melanoma cell line 3 (Minor). The results are summarized in FIG. 7. Referring to FIG. 7, note that the ordinate represents molar concentration (−log 10) and the abscissa percent of radiolabelled leucine uptake. The percent of leucine uptake is a measure of protein synthesis by nucleated cells. The lower the percent of leucine uptake, the less protein synthesis by the cell.

As may be seen from FIG. 7, both the XMMME-001-RTA and XMMME-002-RTA conjugates inhibit protein synthesis in melanoma cells but not in the control cells. A cocktail of approximately equal amounts of XMMME-001-RTA and XMMME-002-RTA conjugates was also studied. Note that the cocktail demonstrated significantly greater inhibition of protein synthesis than either of the conjugates individually.

The killing specificity of the XMMME-001-RTA plus XMMME-002-RTA cocktail was studied in vitro using melanoma cell line 3 and breast carcinoma cell line 11. The results of these studies are summarized in FIG. 8 as a function of percent leucine uptake as in FIG. 7. The cocktail demonstrates a high degree of specificity toward the melanoma cell line with no demonstrable activity toward the breast carcinoma cells.

We claim:

1. A cytotoxic conjugate, comprising a lectin A chain purified with anti-lectin B chain antibodies bound to a monoclonal antibody that binds substantially only human melanoma associated antigen to about 240 kD/480 kD m.w.

2. The conjugate as claimed in claim 1, wherein the monoclonal antibody is that produced by hybridoma XMMME-001 having A.T.C.C. Accession No. HB8759.

3. The conjugate as claimed in claim 1, wherein the monoclonal antibody is that produced by hybridoma XMMME-002 having A.T.C.C. Accession No. HB8760.

4. The conjugate as claimed in claim 1, wherein the lectin A chain is abrin A chain purified with anti-abrin B chain antibodies.

5. A cytotoxic therapeutic cocktail comprising a first conjugate of a lectin A chain purified with anti-lectin B chain antibodies bound to a monoclonal antibody that binds substantially only human melanoma associated antigen of about 240 kD/480 kD m.w. and a second conjugate of a lectin A chain purified with anti-lectin B chain antibodies bound to a different monoclonal antibody that binds substantially only human melanoma associated antigen of about 240 kD/480 kD m.w. than in the first conjugate.

6. The cocktail as claimed in claim 5, wherein the monoclonal antibody of the first conjugate is that produced by hybridoma XMMME-001 having A.T.C.C. Accession No. HB8759.

7. The cocktail as claimed in claim 5, wherein the monoclonal antibody of the first conjugate is that produced by hybridoma XMMME-002 having A.T.C.C. Accession No. HB8760.

8. The cocktail as claimed in claim 6, wherein the monoclonal antibody of the second conjugate is that produced by hybridoma XMMME-002 having A.T.C.C. Accession No. HB8760.

9. The cocktail as claimed in claim 5, wherein the lectin A chain of the first and second conjugates is abrin A chain purified with anti-abrin B chain antibodies.

10. The cocktail as claimed in claim 5, wherein the lectin A chain of the first conjugate is ricin A chain purified with anti-ricin B chain antibodies and the lectin A chain of the second conjugate is abrin A chain purified with anti-abrin B chain antibodies.

11. A method for the treatment of human melanoma cells, comprising administering a therapeutic dose of the conjugate claimed in claim 1 to a human melanoma cell host.

12. A method for the treatment of human melanoma cells, comprising administering a therapeutic dose of the cocktail claimed in claim 5 to a human melanoma cell host.

13. A method for the treatment of human melanoma cells, comprising administering approximately 0.01 to 20.0 milligrams of the conjugate claimed in claim 1 to a melanoma cell host per kilogram of host body weight.

14. A method for the treatment of human melanoma cells, comprising administering the clocktail claimed in claim 5 to a melanoma cell host, wherein the total dose of all conjugates is in the approximate range of 0.01 to 20.0 milligrams per kilogram of host body weight.

15. Antibodies having substantially the same specificity as antibodies produced by hybridoma XMMME-001 having A.T.C.C. Accession No. HB8759 which produces antibodies that bind substantially only human melanoma associated antigens of about 240 kD/480 kD m.w.

16. Antibodies having substantially the same specificity as antibodies produced by hybridoma XMMME-002 having A.T.C.C. Accession No. HB8760 which produces antibodies that bind substantially only human melanoma associated antigens of about 240 kD/480 kD m.w.

17. Hybridoma XMMME-001 having A.T.C.C. Accession No. HB8759 which produces antibodies that bind substantially only human melanoma associated antigens of about 240 kD/480 kD m.w.

18. Hybridoma XMMME-002 having A.T.C.C. Accession No. HB8760 which produces antibodies that bind substantially only human melanoma associated antigens of about 240 kD/480 kD m.w.

19. A cytotoxic conjugate, comprising ricin A chain purified with anti-ricin B chain antibodies bound to a monoclonal antibody that binds substantially only human melanoma associated antigen of about 240 kD/480 kD m.w.

20. A cytotoxic therapeutic cocktail comprising a first conjugate of ricin A chain, purified with anti-ricin B chain antibodies, bound to a monoclonal antibody that binds substantially only human melanoma associated antigen of about 240 kD/480 kD m.w., and a second conjugate of ricin A chain, purified with anti-ricin B chain antibodies, bound to a different monoclonal antibody than in the first conjugate, that binds substantially only human melanoma associated antigen of about 240 kD/480 kD m.w.

* * * * *

Exhibit 4

Scintigraphic Detection of Metastatic Melanoma Using Indium 111/DTPA Conjugated Anti-gp240 Antibody (ZME-018)

By John M. Kirkwood, Ronald D. Neumann, Sami S. Zoghbi, Marc S. Ernstoff, Eugene A. Cornelius, Coralie Shaw, Toni Ziyadeh, Judith A. Fine, and Michael W. Unger

We evaluated the toxicity, pharmacokinetics, and localization of a monoclonal IgG_{2a} murine anti-human melanoma (gp240) antibody (ZME-018) that recognizes a tumor-associated cell surface glycoprotein of 240,000 molecular weight present in most melanomas. The antibody was conjugated with DTPA (diethylenetriamine pentaacetic acid) and labeled by chelation of ^{111}In. One mg of antibody labeled with 5 mCi of ^{111}In was infused, together with 0 to 40 mg of "cold" carrier ZME-018. The blood clearance, urinary excretion, and in vivo localization were determined in 26 patients. Scintigraphic images were obtained at 24 hours and 72 hours in all patients. Mild toxicity occurred in one patient. The half-time clearance of labeled monoclonal murine antibody (MoAb) from the blood increased from 16.1 hours at an antibody dose of 1 mg to 35.9 hours at 40 mg. Males showed faster clearance from the blood than did females or a single castrated male, perhaps due to selective concentration of antibody in the testes. Nonspecific uptake in liver, spleen, bone marrow, and intestine was seen in all patients. The percentage of known metastatic foci detected increased with the total dosage of antibody, from 23% at doses ≤ 5 mg, to 65%, 87%, and 78% for 10, 20, and 40 mg, respectively. We conclude that at doses ≥ 10 mg, ZME-018 is a safe and potentially useful agent for the scintigraphic detection of metastatic malignant melanoma.

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MELANOMA is a disease of increasing incidence for which no therapeutic intervention after primary surgery has a proven survival advantage.[1,2] The optimal use of existing palliative treatments, as well as the application of alternative new investigational strategies, rests heavily upon the early detection of metastatic disease. When metastatic melanoma is at its smallest, it is theoretically most susceptible to immunological, chemotherapeutic, and other systemic interventions.[3] Diagnostic scintigraphic techniques for the detection of metastatic melanoma offer dynamic advantages over purely anatomic, static radiographic techniques, but no highly sensitive and specific means for scintigraphic detection of metastatic melanoma has been developed.[4] A variety of murine monoclonal antibodies (MoAb) against cell-surface and other antigens of melanoma have been developed that detect antigens of different molecular sizes and degrees of restriction to melanoma. Multiple laboratories have thus identified immunologically functional Ia antigens in melanoma,[5-7] and other tumor antigens that appear to represent markers of tissue differentiation.[8,9] Other antigens are markers of potential physiological significance in neoplasia and fetal tissues.[10,11] Gp240 is a melanoma-associated antigen that has exhibited greater restriction to melanoma than other antigens.[12-17] Gp240 has been found in 80% to 94% of melanoma specimens studied in a comparative examination of multiple MoAbs tested against a range of neoplastic and nonneoplastic melanocytic lesions in our laboratory.[18] Immunohistological studies of melanoma and other melanocytic processes with several murine MoAbs, including the antibody ZME-018, have shown the presence of gp240 antigen more frequently than p97 antigen[10] and have prompted our pursuit of this antibody to gp240 for immunoscintigraphic detection of metastatic melanoma.[19]

Here we report phase I-II radioimmunoscintigraphic studies of ZME-018 in which we have

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found no significant toxicity, and preliminary encouraging data regarding the diagnostic capabilities of this antibody administered in escalating doses to 26 subjects with metastatic melanoma. The antibody was covalently coupled to the bifunctional chelator, diethylenetriamine pentaacetic acid (DTPA), and labeled by chelation of ^{111}In for this study. This radiometal avoids the problem of dehalogenation that has occurred with radioiodinated antibodies; and has been reported to yield improved tumor-blood ratios of radioactivity in comparison to radioiodinated antibody.[20-22] Others have reported comparable pharmacokinetics of plasma ^{111}In radioactivity and antibody clearance (determined by enzyme-linked immunoabsorbent assay), with the 96.5 antibody to the melanoma p97 antigen labeled by a similar chelation technique. Unfortunately, this IgG_{2a} antibody to p97 was capable of detecting only approximately 50% of 100 known tumor sites.[21]

Overall, there is clearly a need for the exploration of new imaging methods for the detection of metastatic melanoma, and monoclonal antimelanoma antibodies provide the most refined tools available to achieve this.

MATERIALS AND METHODS

Preparation of ZME-018 MoAb Labeled With ^{111}In

The specificity and distribution of the melanoma-associated antigen gp240 as detected by ZME-018 has been described.[15-17,23] The antibody is an IgG_{2a} murine monoclonal hybridoma product harvested from Balb/c mouse ascites and purified by diethylaminoethyl (DEAE) column chromatography. The antibody was conjugated with the chelating agent, DTPA, using a modification of the method of Krejcarek and Tucker.[24] It was provided in a sterile and nonpyrogenic form, with normal human serum albumin as carrier protein, for radionuclide chelation immediately before clinical use. A uniform dose of 5 mCi ^{111}In was used to avoid the problems reported by others with the use of varying quantities of radionuclide.[21] Antibody reactivity to known gp240-positive cultured melanoma lines (Y Mel 84-420 and Y Mel 81-180) was determined to assure preservation of radiolabeled antibody immunoreactivity in representative DTPA-conjugated ^{111}In chelated antibody preparations. These tests revealed a minimum of 29% to 32% antibody uptake with 6.2 μg antibody in 0.5 mL medium incubated 24 hours at 4°C over 2.0×10^6 melanoma cells. No binding to gp240-negative fibroblast cultures was observed.

Antibody ZME-018, conjugated with DTPA, was provided by Hybritech, Inc, San Diego, in sterile pyrogen-free aliquots (1 mg each). The conjugated antibody was radiolabeled with a buffered solution of ^{111}In. Instant thin-layer chromatography was used as a test to confirm the association of the radioactivity with the MoAb. The labeling efficiency was 94.9% ± 1.56%.

Subjects

Twenty-six subjects with histologically confirmed malignant melanoma and measurable tumor documented by physical examination, or radiographic studies, gave written informed consent to this study. The study protocol was approved by the Yale Human Investigations Committee. Seventeen men and nine women of median age 55 (range, 25 to 87) were enrolled. Table 1 summarizes the demography of the patients, sites of known disease, and quantities of antibody administered. Patients no. 5, 6, and 9 entered this study having had metastatic disease in regional lymph nodes in the past, and each had developed palpable or radiographic lesions suspected of being further recurrence of melanoma. However, subsequent surgical exploration or imaging studies in each failed to confirm active tumor that could have been imaged at the time of antibody study. In addition, patient no. 24 had a single skin metastasis 3 mm in diameter, and did not show evidence of any other metastases on antibody scintigraphy or other restaging studies. Thus, these four patients contributed only to the toxicity and pharmacokinetics analysis of the study.

Patients gave negative histories for prior murine antibody exposure, and had negative intradermal skin test reactions to the antibody solution (1 μg MoAb). Complete medical oncology history, physical examination, EKG, and hematologic/urinary/blood chemistry profiles were obtained in all patients. Documentation of metastatic disease included the enumeration of all disease sites by physical examinations, PA and lateral chest radiographs, tomographic gallium 67 scans, ^{99m}Tc sulfur colloid liver-spleen scans, abdominal ultrasound studies, and computed tomography (CT) brain and body scans. The gallium scans were obtained within 4 weeks of antibody imaging in 13 of the study patients, and within 12 weeks in 17 of the study patients. Where applicable, CT scans were obtained within 4 to 12 weeks as well. The size of metastases was determined by physical examination and on CT scans or radiographs; CT scans were best for this purpose.

Study Methods

Study patients were observed throughout the administration of antibody and for four hours thereafter in the outpatient medical oncology clinic. The radiolabeled 1 mg of MoAb was mixed with a variable quantity of "cold" MoAb, and infused intravenously (IV) in 100 mL 0.9% NaCl solution over one hour. Groups of two to five patients entered each dosage tier as shown in Table 1. Each patient received the MoAb only once. Multiple images of patients were obtained at 24 and 72 hours, and at optional later times, using a gamma camera with computerized image storage (General Electric 400AT and Star Systems, Bridgeport, CT). Patients were clinically examined at a minimum frequency of monthly, with CT and ^{67}Ga performed in most within a month of antibody imaging. Antibody images were scored in a multidisciplinary conference, including medical oncology, radiology, nuclear medicine, and nursing/data management personnel. Sites of uptake were identified by nuclear medicine and scored only if concurred upon by all participants in the multidisciplinary effort.

Table 1. Characteristics of Study Patients and Disease Sites Detected Anti-gp240 by Antibody Imaging

Patient No.	Sex/Age	Ab Dose (mg)	Disease Sites Detected/Known										
			Peripheral Nodes	Soft Tissue	Lungs	Mediastinum and Hila	Abd Nodes	Abd Masses	Liver	Bone	Brain	Total	False-Positives
1	M/87	1.0	2/3							0/1		2/4	1-Bone met MM
2	M/33	1.0	1/3	0/7	0/2			0/1				1/13	0
3	M/59	2.5	1/2									1/2	0
4	M/51	2.5						1/1				1/1	0
5	M/34	2.5										0/0	1-Ax
6	F/55	2.5										0/0	2-Ax, heart
7	M/70	5.0	1/1		0/1							1/2	3-Ax, heart; thyroid
8	M/65	5.0			0/1	0/1						0/2	2-Ax, frontal sinusitis
9	F/62	5.0										0/0	4-Ax, breasts, heart, colostomy stoma
10	M/52	5.0			0/5							0/5	3-Ax, thyroid, heart
11	F/69	5.0		1/1								1/1	2-Ax, knee ligaments
12	F/49	10.0	1/1	1/1	0/1		2/2					4/5	3-Ax, breasts, heart
13	F/33	10.0			0/1	1/2						1/3	2-Ax, heart
14	F/66	10.0	2/2		2/5		1/1	2/3	0/1			7/12	1-Ax
15	F/36	10.0			2/3				1/1			3/4	2-Ax, heart
16	M/59	10.0		1/1	1/1							2/2	1-Ax
17	M/56	20.0	1/1		0/3	0/1						1/5	1-Ax
18	F/36	20.0				1/3		1/1				2/4	2-Ax, breasts
19	M/64	20.0	1/2		0/1	0/1						1/4	0
20	M/75	20.0			1/1							1/1	1-Ax, bone (prostate met)
21	F/72	20.0		54/54		1/1						55/55	0
22	M/60	40.0		1/1			0/2	1/1				2/4	2-Ax, bowel
23	M/25	40.0									1/1	1/1	1-Ax
24	M/51	40.0										0/0	1-Ax
25	M/58	40.0							0/1	2/2		2/3	1-Ax
26	M/41	40.0	5/5	9/12		1/1	1/2	3/3	1/1			20/24	0
Total sites by location			15/19	67/77	6/25	4/10	4/7	8/10	2/4	2/3	1/1	109/156	
Percentile detection			79	87	24	40	57	80	50			70	

Abbreviations; Ab, antibody; Abd, abdominal; met; metastases; Ax, axilla.

Table 2. Antibody Clearance According to Dose Infused

Total* Antibody Dose (mg)	Patients (n)	Clearance From Circulation (T½ h ± SD)
1.0	2	16.1 ± 0.5
2.5	4	19.6 ± 1.9
5.0	5	26.4 ± 5.2
10.0	5	27.4 ± 7.2
20.0	5	30.8 ± 12.9
40.0	5	35.9 ± 7.7

Note. Cumulative urinary excretion in 48 hours is 6.1% ± 2.3% of infused dose of radioactivity.
*Including 1.0 mg labeled DTPA-coupled antibody.

Measurement of Blood Clearance and Urinary Excretion of Radioactivity

Whole blood samples (5 mL) were taken at 0 and 30 minutes, and 2, 3, 24, and 72 hours after antibody infusion. Urine collections were obtained from all patients up to 48 hours postinfusion. Duplicate 100 μL blood and urine samples were counted for radioactivity by scintillation spectrophotometry. The counts were adjusted for the physical decay of ^{111}In, and pharmacokinetics calculated by nonlinear regression analysis.

RESULTS

Pharmacokinetics of ZME-018 Anti-p240 Monoclonal Antibody Labeled With ^{111}In

Table 2 shows the whole blood clearance and urinary excretion of radionuclide label according to the dose of antibody infused.

Toxicity

One patient demonstrated mild toxicity. She had a history of allergies and asthma. Her preliminary skin test was negative. Approximately 90 minutes after the infusion was begun, she complained of headache, nasal congestion, and mild wheezing. A mild rash developed. Benadryl (Park-Davis, Morris Plains, NJ) 25 mg, per os, resulted in complete relief.

Radioimmunoscintigraphy Results

The distribution of known tumor sites ≥ 1 cm in diameter is given in Table 1. The disease in this population is representative of the population with metastatic melanoma followed in the Yale Melanoma Unit. It is notable that an increasing fraction of known sites of disease was detected as the total dosage of antibody was increased from 1 mg to 40 mg (Table 3). The overall sensitivity for the entire study was 109 of 157 metastatic foci, or 70%. For the 2.5 mg dose of antibody, only three tumor sites were studied, so the data at this dose tier are unreliable. However, for all antibody dosages ≤5 mg, the sensitivity was seven of 30 or 23%. Above this dosage, there was an increase in sensitivity, but this was not significantly different among the 10, 20, and 40 mg doses. The pooled data for these three groups (10, 20, and 40 mg) gave a sensitivity of 102 of 127 or 80% of known sites of 1 cm diameter or greater. In patient no. 21, the large number of lesions and the high sensitivity of detection may bias the sensitivity data for the 20-mg group.

Patients no. 4, 21, and 26 were found to have multiple previously unknown tumor sites in abdomen and soft tissue, which were confirmed by subsequent clinical or imaging examinations. Numerous foci of previously unknown disease in patients no. 21 and 26 were detected at the limit of standard imaging techniques. These are not included in the tables, since they were confirmed by later evolution or by ^{67}Ga studies and were thus of unknown exact size at the time of ZME-018 imaging (See Figs 1 and 2).

Table 3. Summary of Tumor Detected by ZME-018 ^{111}In According to Dosage of Antibody Infused

MoAb Dose (mg)	Patients (n)	Total of Known Tumor* Sites	True-Positive	False-Positive	False-Negative	Overall Sensitivity (%)
1.0	2	17	3	1	14	18
2.5	4	3	2	3	1	67
5.0	5	10	2	14	8	20
10.0	5	26	17	9	9	65
20.0	5	69	60	4	9	87
40.0	5	32	25	5	7	78
						(x=70)

*Sites of ≥ 1 cm.

The sensitivity of ZME-018 antibody imaging according to the location of the metastases is presented in Table 1. Metastases in soft tissue, such as skin, subcutaneous tissue, and muscle, were most accurately detected (87%). Seventy-nine percent of peripheral lymph node foci of tumor were detected. Lung metastases were poorly seen (25%). The numbers for other individual anatomic areas are smaller and less reliable. However, as data for all abdominal sites are pooled, 14 of 21 or 67% were detected.

The antibody localized nonspecifically in liver, spleen, bone marrow, and intestine of all patients, and to the axillae of a majority. The testes of all noncastrate males demonstrated preferential uptake, without relation to the presence of tumor. The sites of false-positive uptake are listed in Table 2. These were not a source of serious confusion with tumor, except for the colostomy stoma (no. 9) and thyroid (no. 7) uptake. Cardiac uptake occurred in seven patients (no. 6, 7, 9, 10, 12, 13, and 15), and was differentiated from tumor by concurrent and follow-up chest radiographs, CT scans, and ultrasound.

DISCUSSION

The present phase I-II radioimmunoscintigraphy study of the murine MoAb ZME-018, directed against the melanoma-associated glycoprotein gp240, presents the first pharmacokinetic and imaging results with this ^{111}In-DTPA labeled MoAb. Previous in vitro immunohistological studies with anti-gp240 MoAb have compared favorably with parallel studies of the MoAb 96.5 anti-p97, the most extensively studied imaging agent for metastatic melanoma.[10,11,13-16,18,21-23] In our studies, there was a high and consistent radioactive labeling of antibody. There was also acceptable binding of this radiolabeled antibody to a gp240-positive tumor cell line, Y Mel 84-420, indicating preservation of antibody reactivity. Previous major studies of the imaging sensitivity of radiolabeled antibody to melanoma antigens have involved ^{111}In labeling of the 96.5 antibody to the p97 antigen, by means of DTPA chelation.[24,33] One milligram of radiolabeled antibody was mixed with increasing amounts of cold antibody, providing a total MoAb dosage range of 0.5 to 20.0 mg. Overall, 50 of 100 metastatic foci were detected (sensitivity, 50%). In our study, the overall sensitivity was 109 of 157 (69%). In the 96.5 study, sensitivity for an antibody dose $\leq$5 mg was 25%, and in our study 23%. For a total 96.5 antibody dose of 10 and 20 mg, sensitivity was 68%, whereas in our pooled 10-, 20-, and 40-mg groups, sensitivity was 81%. The ^{111}In-96.5 study used only 2.0 to 2.5 mCi of ^{111}In, except for one test site. That group received 5.0 mCi at a total MoAb dose of 20 mg; sensitivity was 81%, whereas in our comparable dosage tier, the sensitivity was 87% (Table 3). Thus, our results confirm that imaging with increased doses of cold antibody may demonstrate a greater proportion of metastatic foci. Our results are also encouraging in comparison to results reported with directly radioiodinated antibodies, and F(ab')$_2$ fragments labeled with ^{99m}Tc, ^{111}In, ^{123}I, or ^{131}I.[25-28]

A ten-center study of ^{111}In and ^{99m}Tc-labeled F(ab')$_2$ fragments of an antibody similar to the high molecular weight antigen of melanoma has been recently published. This work employed no standard corollary imaging techniques for disease confirmation and variably employed "subtraction" methods due to high background uptake of ^{99m}Tc. The authors conclude that F(ab')$_2$ labeled with ^{99m}Tc may be superior to F(ab')$_2$ labeled with ^{111}In, but report detection of 94 of 159 lesions (59%) of various sizes using 5 mCi ^{111}In with 2 mg F(ab')$_2$. While we have detected 109 of 156 lesions (70%) in the study of intact anti-p240 ^{111}In, the differences due to larger quantity of intact antibody here preclude simple comparison with the results of Siccardi et al.[29] It is notable that 2 mg of F(ab')$_2$ labeled with 10 to 30 mCi ^{99m}Tc detected 74% of known tumor masses in that study, whereas the use of anti-p240 labeled with 1 mCi ^{111}In and 10, 20, or 40 mg cold antibody here allowed detection of up to 78% to 87% of tumor sites. A correlation between lower stage of disease and imaging success was not apparent in this study, where lesions imaged were all detected in stage III to IV patients. If the findings of the multicenter trial reported by Siccardi are valid, one may project improved results of the anti-p240 antibody in stage II patients, arguing for earlier use of this diagnostic modality.

This study has demonstrated localization of the anti-p240 antibody in a sufficient number of tumor foci in skin, subcutaneous tissues, muscle, and peripheral lymph nodes to suggest a possible

future clinical utility of this technique for disease detection in these areas. There is a critical need to improve the precision of staging, and specifically, the assessment of regional lymph nodes of patients at high risk of relapse. Immunoscintigraphy with anti-p240 provides an avenue by which regional lymph node status might be assessed preoperatively. If successful in a histologically corroborated trial, immunoscintigraphy would provide a means to focus surgical intervention for lymphadenectomy in the highest-risk patient category. While numerous tumor foci that were missed by standard methods of detection were also demonstrated, detection of these sites was not the objective of our present study, and requires surgical-pathologic confirmation that we

Fig 1. (A and B) See legend on facing page.

Fig 1. Patient no. 21: tomographic ^{67}Ga images ([A] anterior and [B] posterior), compared with anti-gp240 ^{111}In conjugate images of (C) chest, and upper and lower abdomen, and (D) arms, legs, and thighs. Multiple lesions apparent on antibody image were inapparent on gallium scan, but palpable.

did not undertake during the present antibody study.

A high incidence of axillary uptake in this study may be due to localization in hair bulbs, sweat, or sebaceous glands, as suggested in recent immunochemical studies.[30] In general, the differentiation of false- from true-positives involved a careful correlation of physical examination and imaging studies, especially CT and ^{67}Ga during patient follow-up evaluation. The protocol for scoring of antibody images (as of all tumor imaging studies) required consensus of a team of radiology, nuclear medicine, and medical oncology physicians. This system assured consistency and quality control.

Analysis of the pharmacokinetics of MoAb 96.5 revealed a decreasing volume of distribution at higher doses, and decreased localization in liver.[21] However, it was noted that blood clearance fit an open one-compartment mathematical model, with a T½ in plasma of 31 hours independent of dosage. The study of 96.5 MoAb also revealed no differences between male and female blood clearances, in contrast to our anti-p240 study. Two findings in our study merit discussion: first, the prolonged plasma clearance T½ values observed with increasing total antibody dosages parallel the improved sensitivity for detecting metastatic sites; second, the differences in male and female blood plasma clearance values suggest the presence of a tissue "sink" that is associated with gender, ie, the testes. Imaging studies that showed localization to the testes of male patients suggest to us that testicular tissue binding, rather than hormonal effect of testicular origin, may explain some of the differential phar-

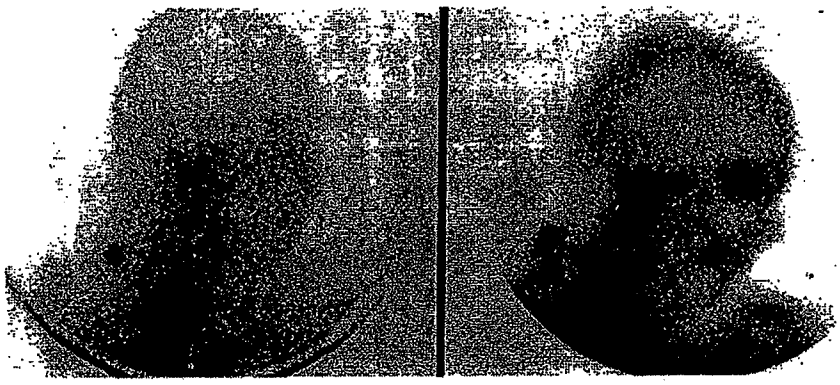

Fig 2. Patient no. 26: (left) gallium and (right) gp240 images of neck and supraorbital soft tissue metastases.

macokinetics observed in this study. Our one study carried out in a castrated male patient with melanoma and a history of prostatic carcinoma, in whom blood clearance T½ values resembling the female pattern were observed, tends to confirm this hypothesis. However, preliminary immunohistological studies (data not shown) suggest only minimal stromal binding of ZME-018 in human testicular tissue.

We conclude that the monoclonal antibody ZME-018, radiolabeled with 5.0 mCi of ^{111}In, is a generally nontoxic immunoscintigraphic reagent that is capable of imaging most metastatic melanoma sites of ≥ 1 cm in size. Comparative analysis of the imaging of melanoma by ^{67}Ga citrate and other antibodies, with detailed biodistribution data, will be published in two subsequent reports.[31,32] Future efforts to improve antibody imaging and therapy of melanoma are in progress, with attention to reduction of background uptake and induction of increased expression of the relevant tumor antigens (using cytokines such as the interferons) in conjunction with antibody administration.

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Exhibit 5

United States Patent [19]

Stevens et al.

[11] **Patent Number: 4,894,227**

[45] **Date of Patent: * Jan. 16, 1990**

[54] **COMPOSITION OF IMMUNOTOXINS WITH INTERLEUKIN-2**

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[73] Assignee: **Cetus Corporation,** Emeryville, Calif.

[*] Notice: The portion of the term of this patent subsequent to Sep. 5, 2006 has been disclaimed.

[21] Appl. No.: **55,681**

[22] Filed: **May 29, 1987**

Related U.S. Application Data

[63] Continuation-in-part of Ser. No. 892,596, Aug. 1, 1986.

[51] **Int. Cl.**[4] **A61K 37/02**

[52] **U.S. Cl.** **424/85.2;** 424/85.1; 424/85.8; 424/85.91; 514/2; 514/8; 514/21; 514/885; 530/351; 530/389; 530/391

[58] **Field of Search** 424/85, 85.1, 85.2, 424/85.8, 85.91; 514/2, 8, 21, 885; 530/351, 389, 391

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[57] **ABSTRACT**

Anti-tumor activity in humans can be augmented by administering to the mammalian host a pharmacologically effective amount of mammalian IL-2 and at least one immunotoxin that binds selectively to human tumor cells. The IL-2 and immunotoxin are preferably administered separately to the host. The composition is useful for prophylactic or therapeutic treatment of such cancers as ovarian and breast cancer.

10 Claims, No Drawings

COMPOSITION OF IMMUNOTOXINS WITH INTERLEUKIN-2

This application is a continuation-in-part application of copending U.S. Ser. No. 892,596 filed Aug. 1, 1986.

BACKGROUND OF THE INVENTION

1. Field of the Invention

This invention relates to use of a combination of interleukin-2 and an immunotoxin directed against tumor cells in the therapeutic or prophylactic antitumor treatment of mammals using simultaneous or alternate administration of these components.

2. Background Art

Interleukin-2 (IL-2), a lymphokine produced by normal peripheral blood lymphocytes that induces proliferation of antigen or mitogen stimulated T cells after exposure to plant lectins, antigens, or other stimuli, was first described by Morgan, D. A., et al., *Science* (1976), 193:1007–1008. Then called T cell growth factor because of its ability to induce proliferation of stimulated T lymphocytes, now interleukin-2 is recognized as modulating a variety of functions of immune system cells in vitro and in vivo.

IL-2 was initially made by cultivating human peripheral blood lymphocytes (PBL) or other IL-2-producing cell lines. See, for example, U.S. Pat. No. 4,401,756 Recombinant DNA technology has provided an alternative to PBLs and cell lines for producing IL-2. Taniguchi, T. et al., *Nature* (1983), 302:305–310 and Devos, R., *Nucleic Acids Research* (1983), 11:4307–4323 have reported cloning the human IL-2 gene and expressing it in microorganisms.

U.S. Pat. No. 4,518,584 describes and claims muteins of IL-2 in which the cysteine normally occurring at position 125 of the wild-type or native molecule has been replaced with a neutral amino acid, such as serine or alanine Copending U.S. application Ser. No. 810,656 filed Dec. 17, 1985 discloses and claims an oxidation-resistant mutein such as IL-2 which is biologically active wherein a methionine residue of the parental protein which is susceptible to chloramine T or peroxide oxidation is replaced with a conservative amino acid such as alanine. The above IL-2 muteins possess the biological activity of native IL-2. U.S. Pat. Nos. 4,530,787 and 4,569,790 disclose and claim methods for purifying recombinant native IL-2 and muteins thereof, as well as the purified form of IL-2.

U.S. Pat. No. 4,604,377 issued Aug. 5, 1986 discloses an IL-2 composition suitable for reconstituting in a pharmaceutically acceptable aqueous vehicle composed of oxidized microbially produced recombinant IL-2. The IL-2 is noted as useful in combination with cytotoxic chemotherapy or irradiation or surgery in the treatment of malignant or pre-malignant diseases in a direct therapeutic or adjuvant setting or in combination with other immune-modulating drugs, lymphokines (e.g., IL-1, IL-3, CSF-1 and IFNs), or naturally occurring or inducible anti-cellular toxins.

Rosenberg and his coworkers have shown that systemic administration of recombinant IL-2 in high doses causes regression of established metastatic cancers in mice (Rosenberg et al., *J. Exp. Med.* (1985) 161:1169–1188); and, in conjunction with lymphokine-activated killer cells (Rosenberg, S. et al., *New Eng. J. Med.* (1985), 313:1485–1492), and tumor-infiltrating lymphocytes (Rosenberg et al., *Science* (1986) 233:1318–1321), in humans.

Since the mid-1970s, there have been numerous reports of murine monoclonal antibodies that interact with human breast cancer associated antigens. In these reported studies, mice were immunized and boosted with human milk fat globule proteins, breast cancer cell lines or breast cancer membrane extracts. Immune splenocytes were fused with mouse myeloma cells and hybridomas were selected based on some specificity of the culture supernatant for breast or breast cancer antigens. Taylor-Papadimitriou, J. et al., *Int. J. Cancer* (1981) 28:17–21; Yuan, D., et al., *JNCI* (1982) 68:719–728; Ciocca, D. R. et al., *Cancer Res.* (1982) 42:4256–4258.

More recently, investigators at Cetus Corporation have discovered murine monoclonal antibodies that bind selectively to human breast cancer cells, are IgGs or IgMs, and, when conjugated to ricin A chain to form an immunotoxin, exhibit a tissue culture inhibitory dose which results in 50% of control (untreated) protein synthesis (TCID 50%) at immunotoxin concentrations of less than about 10 nM against at least one of MCF-7, CAMA-1, SKBR-3, or BT-20 cells. These antibodies are described more fully in EPC Patent Publication No. 153,114 published Aug. 28, 1985, the disclosure of which is incorporated herein by reference.

In addition, researchers at Cetus Corporation have discovered murine monoclonal antibodies which do not bind to blood cells, have a breast tumor binding range of at least 0.25 (i.e., they bind to at least 25% of breast tumors tested) or have a breast cancer cell line binding range of greater than or equal to 0.25, have a normal tissue reactivity as defined below for human breast and/or ovarian cells equal to or less than 0.09, are IgGs or IgMs, and, when conjugated to an imaging moiety, produce a signal sufficient to image breast cancer tumors. These antibodies include most of those described above and are described more fully in European Pat. Pub. No. 220,858 published May 6, 1987, the disclosure of which is incorporated herein by reference.

Immunotoxins, which are comprised of an antibody conjugated to a toxin, have been used for therapy of various cancers to which the antibody is specific. Certain immunotoxin molecules may be too large to reach the tumor cells efficiently due to poor diffusion out of capillaries.

Combination chemotherapy using two or more anticancer drugs to treat malignant tumors in humans is currently in use in research and in the clinic. The anticancer drugs may be antimetabolites, alkylating agents, antibiotics, general poisons, etc. Combinations of drugs are administered in an attempt to obtain a synergistic cytotoxic effect on most cancers, e.g., carcinomas, melanomas, lymphomas and sarcomas, and to reduce or eliminate emergence of drug-resistant cells and to reduce side effects of each drug.

Dr. Rosenstein et al., *J. Immunol.* (1986) 137:1735–1742 disclosed that IL-2 increases the vascular permeability and rate of serum albumin diffusion into organs. Lotze et al., *J. Immunol.* (1985) 135:2865 disclosed that reversible fluid retention problems result from IL-2 administration.

To applicants' knowledge no one has administered immunotoxin and IL-2 to decrease or eliminate tumor burden

SUMMARY OF THE INVENTION

Accordingly, the present invention provides a composition suitable for parenteral or subcutaneous administration to a warm-blooded mammalian host for therapeutic or prophylactic treatment of tumor burden comprising a mixture, in pharmacologically effective amounts, of IL-2 from a mammalian species and at least one immunotoxin that binds selectively to cells containing the tumor burden.

In another aspect, the invention provides a method for therapeutic or prophylactic treatment of tumor burden in a warm-blooded mammalian host comprising administering to said host a pharmacologically effective amount of a combination of IL-2 from a mammalian species and at least one immunotoxin that binds selectively to cells containing the tumor burden.

Preferably the IL-2 is a recombinant human IL-2, and the monoclonal antibody employed in the immunotoxin selectively binds to human breast and/or ovarian cancer cells and has a G or M isotype, and the tumor burden line treated is breast and/or ovarian cancer.

The combination of IL-2 and immunotoxin(s) in pharmacologically effective amounts is expected to provide suitable treatment of a variety of forms of cancer, especially breast and ovarian cancers.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The term "therapeutic" treatment refers to administration to the mammalian host or patient of the IL-2 and immunotoxin(s) after the patient has developed cancer, (i.e., after a tumor burden has been determined), as measured by any means in the art, with resultant decrease or elimination of the existing tumor burden.

The term "propylactic" treatment refers to such administration to prevent recurrence of the cancer after therapeutic treatment has been administered.

The terms "cancer" and "tumor burden" refer to any neoplastic disorder, including such cellular disorders as, for example, renal cell cancer, Kaposi's sarcoma, chronic leukemia, breast cancer, sarcoma, prostate, pancreatic endometrial, and ovarian carcinomas, rectal cancer, throat cancer, melanoma, colon cancer, bladder cancer, mastocytoma, lung cancer and gastrointestinal or stomach cancer. In the method of the invention, the target tumor burden is advantageously breast and/or ovarian cancer.

The term "pharmacologically effective amount" refers to the total amount of each active component of the method or composition herein that is sufficient in showing a meaningful patient benefit, i.e., prolongation of life and/or reduction of disease. When the effective amounts defined herein are employed, more efficacy is obtained using the combination than using either component alone. As applied to an individual active ingredient administered alone, the term refers to that ingredient alone; when combinations are used, the term refers to combined amounts in the preparation that result in the therapeutic or prophylactic effect.

The term "recombinant" refers to IL-2 produced by recombinant DNA techniques wherein generally the gene coding for the IL-2 is cloned by known recombinant DNA technology. For example, the human IL-2 gene is inserted into a suitable DNA vector such as a bacterial plasmid, preferably an *E. coli* plasmid, to obtain a recombinant plasmid, and the plasmid is used to transform a suitable host. The gene is expressed in the host to produce the recombinant protein. Examples of suitable recombinant plasmids for this purpose include pBR322, pCR1, pMB9 and pSC1. The transformed host may be prokaryotic or eukaryotic, including mammalian, yeast, Aspergillus and insect cells. One preferred embodiment herein, but not the only preferred embodiment, employs bacterial cells as the host.

The term "pharmaceutically acceptable" refers to a carrier medium that does not interfere with the effectiveness of the biological activity of the active ingredients and that is not toxic to the hosts to which it is administered.

The term "immunotoxin" as used herein refers to a conjugate of an antibody or fragment of an antibody and a cytotoxic moiety. The antibody or fragment thereof employed must bind selectively to human tumor cells and be effective in an immunotoxin. The antibody is chosen from those described hereinafter if it is effective in the conjugated form. The cytotoxic moiety of the immunotoxin includes a cytotoxic drug or an enzymatically active toxin of bacterial or plant origin or an enzymatically active fragment ("A chain") of such a toxin. Examples of enzymatically active toxins and fragments thereof include diphtheria A chain, nonbinding fragments of diphtheria toxin, exotoxin A chain (from *Pseudomonas aeruoinosa*), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, *Aleurites fordii* proteins, dianthin proteins, *Phytolacca americana* proteins (PAPI, PAPII, and PAP-S), momordica charantia inhibitor, curcin, crotin, saponaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, and enomycin. Ricin A chain, nonbinding active fragments of diphtheria toxin, abrin A chain, and PAPII are preferred. Most preferred is the ricin A chain.

As used herein, the term "selective binding to human tumor cells" refers to preferential binding of the antibodies of the immunotoxin to human cells that are cancerous or exhibit cancerous growth or other properties characteristic of cancer. The antibodies of the immunotoxins do not preferentially bind to normal healthy cells. Examples of such tumor cells include leukemia cells, prostrate cancer cells, colorectal cancer cells, breast cancer cells, ovarian cancer cells, rectal cancer cells, throat cancer cells, melanoma cells, colon cancer cells, bladder cancer cells, lung cancer cells, and gastrointestinal or stomach cancer cells. Most preferably, the antibodies of the immunotoxins selectively bind to breast and/or ovarian cancer cells, as opposed to binding to normal, non-cancerous cells.

As used herein with respect to the exemplified monoclonal anti-human breast cancer antibodies of the immunotoxins, the term "functional equivalent" means a monoclonal antibody that: (a) has a breast tumor binding range of at least 0.25 or has a breast cancer cell line range of greater than or equal to 0.25; (b) binds selectively to human breast cancer cells; (c) has a G or M isotype; and (d) binds to the same antigen or epitope as the exemplified monoclonal antibody, as determined by immunoprecipitation or crossblocking and sandwich immunoassay.

As described above, the term "functional equivalent" as used herein includes four criteria. The last of these criteria, binding to the same antigen or epitope as an exemplified monoclonal antibody, may be demonstrated by experiments which show crossblocking of an exemplified monoclonal antibody by the functionally equivalent monoclonal antibody. Crossblocking occurs as a result of an antibody binding to the same epitope on an

antigen as that bound by one of the exemplified antibodies, or as a result of an antibody binding to a different epitope which is so closely situated on the same antigen that binding of an antibody to one epitope blocks the binding of an antibody to the second epitope. Cross-blocking thus is one of the criteria by which one can determine that a functionally equivalent monoclonal antibody binds to the same antigen or epitope as an exemplified monoclonal antibody.

So-called "sandwich" assays are another method for determining whether an antibody of the immunotoxin binds the same antigen or epitope. In these assays, a first monoclonal antibody is bound to a support, for example, the surface of a microtitre plate well. After treatment to prevent nonspecific binding, a solubilized antigen preparation is added to the bound antibody. Subsequently, a second antibody, having a detectable label, for example, a chromogenic enzyme, is added. If the second antibody binds to the antigen, a different epitope specificity or multiple copies of the same epitope on the same antigen are indicated. If the second antibody fails to bind, either the same epitope specificity or different but proximal antigen specificity is indicated. The results of both the crossblocking and sandwich assay are further defined by a second series of tests such as immune precipitation or Western blotting to characterize the molecular weight(s) of the antigen(s) bound by both antibodies.

The American Type Culture Collection (ATCC), Rockville, Md. USA has a wide variety of cell lines on deposit which produce monoclonal antibodies to target tumors. For example, cell lines producing monoclonal antibodies to human non-small cell lung cancer include 703D4 (deposited as ATCC No. HB8301). Cell lines producing monoclonal antibodies to human melanoma cells include 704A1 (deposited as ATCC No. HB8302). Cell lines producing monoclonal antibodies to small cell carcinoma include the cell lines deposited as ATCC HB8462 and ATCC HB8711. Cell lines producing antibodies to pancreatic carcinoma of ductal origin include the hybridoma deposited as ATCC HB8504. A cell line producing antibodies which bind to an epitope present on adenocarcinomas of the stomach, colon, and pancreas, and to esophagus, breast and ovarian tumors, known as CSLEX1, is deposited as ATCC HB8580.

Conjugates of the antibody and cytotoxic moiety may be made using a variety of bifunctional protein modifying reagents. Examples of such reagents include N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP), iminothiolane (IT), bifunctional derivatives of imidoesters such as dimethyl adipimidate·HCl, active esters such as disuccinimidyl suberate, aldehydes such as glutaraldehyde, bis-azido compounds such as bis (p-azidobenzoyl) hexanediamine, bis-diazonium derivatives such as bis-(p-diazonium-benzoyl)-ethylenediamine, diisocyanates such as tolylene-2,6-diisocyanate, and bis-active fluorine compounds such as 1,5-difluoro-2,4-dinitrobenzene.

The method of this invention involves administering to a warm-blooded mammalian host, including a mouse, rat, rabbit, primate, pig or human host, preferably a human patient, a pharmacologically effective amount of IL-2 and one or more immunotoxins that selectively bind to human tumor cells. The IL-2 and immunotoxin(s) may be combined in vitro before administration if neither is adversely affected chemically and both remain efficacious. Preferably, however, they are separately administered to the patient, in either order or simultaneously. An example is the protocol set forth in Examples 1 and 2 hereof, wherein the IL-2 and immunotoxin are administered separately.

The administration(s) may take place by any suitable technique, including parenteral administration. Examples of parenteral administration include intravenous, intraarterial, intramuscular, subcutaneous, and intraperitoneal, with intravenous, intramuscular and intraperitoneal administration being preferred.

As an example, the patient/host may be treated locally (as by peritumor or intramuscular injection) or systemically with a preparation having IL-2 activity until the capillaries begin to leak large proteins (about 6 days). Then the immunotoxin may be administered with or without the IL-2 for the prescribed duration of treatment. Alternatively, immunotoxin can be administered from day 1 of treatment. Local treatment with IL-2 to promote immunotoxin action may be followed with systemic (e.g., intraperitoneal or intravenous bolus) administration of IL-2 and immunotoxin.

The dose and dosage regimen will depend on whether the IL-2 and immunotoxin(s) are being administered separately or as a mixture, the type of immunotoxin(s) and cancer, the patient/host and the patient's history. The amount must be effective to achieve some tumor reduction or augmentation of LAK activity. The doses may be single doses or multiple doses. If multiple doses are employed, as preferred, the frequency of administration will depend, for example, on the type of component, cancer, dosage amounts, host, etc. For some types of cancers, daily administration may be effective, whereas for other types of cancer, administration every other day or every third day may be effective, but daily administration ineffective. The practitioner will be able to ascertain from clinical trials which route of administration and frequency of administration are most effective in humans in any particular case.

The dosage amount which appears to be most effective herein is one which results in regression in size of the tumor or complete disappearance or non-reappearance of the tumor, and is not toxic or is acceptably toxic to the host. Generally, such conditions as fever, chills and general malaise are considered acceptable. This optimum dose level will depend on many factors, for example, on the type of host and type of cancer, route, schedule and sequence of administration, existing tumor burden, the type of IL-2 and immunotoxin(s), and the definition of toxicity.

Toxicity to the host may be defined by the extent and type of side effects, with fever, chills and general malaise considered acceptable toxicity for the study herein, or sometimes by the amount of body weight gain or by death after a certain period of time. The reversible fluid retention in the body resulting from IL-2 administration is disclosed by Lotze, et al., *J. Immunol.*, 135:2865 (1985). If body weight gain is the criterion for toxicity, typically a gain of from 10 to 20% by weight will be tolerated, with greater than 20% gain being considered toxic.

If there is acceptable toxicity, and if there is pretreatment with recombinant, microbially produced IL-2 and/or concurrent administration daily for 14 days of the IL-2 and for 7 days of immunotoxin beginning at day 1 post-treatment, the dosage level of each administration of immunotoxin made with the anti-breast cancer antibody is 25 to 500 μg/kg of host weight of immunotoxin. The IL-2 level is that given above. If the host

is immuno-compromised (i.e., nude mice, which have a genetic deficiency), the maximum tolerated dose may be lower.

In one preferred embodiment the IL-2 is given at the maximum tolerated dose daily for one week followed by giving one-half the maximum tolerated dose of IL-2 with concomitant administration of the maximum tolerated dose of immunotoxin.

For parenteral administration the IL-2 and immunotoxin(s) will generally each be formulated in a unit dosage injectable form (solution, suspension, emulsion), preferably in a pharmaceutically acceptable carrier medium that is inherently non-toxic and non-therapeutic. Examples of such vehicles include saline, Ringer's solution, dextrose solution, mannitol, and normal serum albumin. Nonaqueous vehicles such as fixed oils and ethyl oleate may also be used. The carrier medium may contain minor amounts of additives such as substances that enhance isotonicity, solubility, and/or chemical stability, e.g., buffers, detergents, and preservatives. The IL-2 and immunotoxin(s) will typically each be formulated in such carriers at a concentration of about 0.1 mg/ml to 100 mg/ml, preferably 0.2 to 1 mg/ml.

Alternatively, the IL-2 and immunotoxin(s) may be made into a sterile, stable lyophilized formulation in which the purified IL-2 and immunotoxin(s) are admixed with a water-soluble carrier such as mannitol, which provides bulk, and about 500 μg of a surfactant such as sodium dodecyl sulfate per mg of IL-2 or 0.01–0.05% in typical formulations to ensure the solubility of the recombinant IL-2 in water, if the immunotoxin is still active at such concentrations. The formulation is suitable for reconstitution in aqueous injections for parenteral administration and it is stable and well-tolerated in the mammalian host, particularly in human patients. The IL-2 formulation method is more completely described in U.S. Pat. No. 4,604,377 issued Aug. 5, 1986, the disclosure of which is incorporated herein by reference.

In an alternative IL-2 formulation, described in copending U.S. application Ser. No. 866,459, filed May 21, 1986, now abandoned, the disclosure of which is incorporated herein by reference, the IL-2 may be solubilized, not by a detergent, but by reacting the IL-2 with an activated polymer selected from polyethylene glycol homopolymers and polyoxyethylated polyols, said polymer having a molecular weight of from 300 to 100,000 daltons, preferably 350 to 40,000 daltons. The polymer is activated by conjugation with a coupling agent having terminal groups reactive with both the free amino or thiol groups of the IL-2 and the hydroxyl group of the polymer. Examples of such coupling agents include hydroxynitrobenzene sulfonic ester, cyanuric acid chloride, and N-hydroxysuccinimide. This modification eliminates the necessity of adding detergents to solubilize the IL-2 at physiological pH. The IL-2 is then formulated directly with the water-soluble carrier and buffer as described above, and the formulation may be lyophilized and the lyophilized mixture reconstituted as described above.

As mentioned above, it is preferred not to admix the components, but rather to administer them separately. If the formulation contains two or more of the components, the relative amounts of each may vary within the ranges described above depending on the efficacy obtained.

The IL-2 herein may be any IL-2 prepared from tissue cultures or by recombinant techniques, and from any mammalian source such as, e.g., mouse, rat, rabbit, primate, pig, and human. Preferably the IL-2 is from a human source. More preferably the IL-2 is recombinant.

The recombinant IL-2 may be obtained as described by Taniguchi et al., *Nature*, 302:305–310 (1983) and Devos, *Nucleic Acids Research*, 11:4307–4323 (1983) by cloning the native human IL-2 gene and expressing it in transformed microorganisms. It may also be an IL-2 mutein as described in U.S. Pat. No. 4,518,584, in which the cysteine normally occurring at position 125 of the wild-type or native molecule has been replaced by a neutral amino acid such as serine or alanine, or an IL-2 mutein as described in copending U.S. application Ser. No. 893,186 filed Aug. 5, 1986, now abandoned, the disclosure of which is incorporated herein by reference, in which the methionine normally occurring at position 104 of the wild-type or native molecule has been replaced by a neutral amino acid such as alanine.

In one embodiment, the IL-2 is an unglycosylated protein which is produced by a microorganism which has been transformed with the human cDNA sequence or a modified human cDNA sequence of IL-2 which encodes a protein with an amino acid sequence at least substantially identical to the amino acid sequence of native human IL-2, including the ability to form the disulfide bond between the cysteines at positions 58 and 105, and has biological activities which are common to native human IL-2. The IL-2 may also be produced from yeast or other hosts, as described above. Substantial identity of amino acid sequences means the sequences are identical or differ by one or more amino acid alterations (deletions, additions, substitutions) which do not cause an adverse functional dissimilarity between the synthetic protein and native human IL-2. Examples of IL-2 proteins with such properties include those described by Taniguchi et al., *Nature* (1983), 302:305–310; Devos, *Nucleic Acids Research* (1983), 11:4307–4323; and by European Patent Publication Nos. 91,539 and 88,195; in U.S Pat. No. 4,518,584, supra, and in copending U.S. application Ser. No. 810,656 filed Dec. 17, 1985, supra. Most preferably, the IL-2 is the des-ala$_1$-IL-2$_{ser125}$ mutein in which the N-terminal alanine of the native IL-2 is deleted and the cysteine at position 125 of the native IL-2 is replaced by a serine residue, the des-ala$_1$-IL-2 ala$_{104}$ser$_{125}$ mutein in which the methionine at position 104 of the native IL-2 is replaced by an alanine residue and the cysteine at position 125 is replaced by a serine residue, or IL-2 wherein any combination of up to five of the first five N-terminal amino acid residues are deleted.

The IL-2 may be produced and purified to clinical purity by the method described and claimed in U.S. Pat. No. 4,569,790, issued Feb. 11, 1986, the disclosure of which is incorporated herein by reference.

The antibodies useful herein are produced from hybridomas prepared from antibody-producing fusion partners. Such fusion partners are generated by immunizing mice with live human cancer cells, such as breast cancer cells, or membrane extracts made therefrom. The mice are inoculated intraperitoneally with an immunogenic amount of the cells or extract and then boosted with similar amounts of the immunogen. Spleens are collected from the immunized mice a few days after the final boost and a cell suspension is prepared therefrom for use in the fusion.

Hybridomas are prepared from the splenocytes and a murine tumor partner using the general somatic cell hybridization technique of B. Kohler and C. Milstein, *Nature* (1975) 256:495–497 as modified by Buck, D. W. et al., In Vitro (1982) 18:377–381. Available murine myeloma lines, such as those from the Salk Institute, Cell Distribution Center, San Diego, Calif., USA, may be used in the hybridization. Basically, the technique involves fusing the tumor cells and splenocytes using a fusogen such as polyethylene glycol. After the fusion the cells are separated from the fusion medium and grown in a selective growth medium, such as HAT medium, to eliminate unhybridized parent cells. The hybridomas are expanded, if desired, and supernatants are assayed for anti-human cancer activity by conventional immunoassay procedures (e.g., radioimmunoassay, enzyme immunoassay, or fluorescence immunoassay) using the immunizing agent (cancer cells or membrane extract) as antigen. Positive clones are characterized further to determine whether they meet the criteria of the antibodies herein, i.e., whether they selectively bind to human tumor cells.

Hybridomas that produce such antibodies may be grown in vitro or in vivo using known procedures. The monoclonal antibodies may be isolated from the culture media or body fluids, as the case may be, by conventional immunoglobulin purification procedures such as ammonium sulfate precipitation, gel electrophoresis, dialysis, chromatography, and ultrafiltration, if desired.

The preferred monoclonal antibodies for the immunotoxins herein bind selectively to human breast and/or ovarian cancer cells, and, therefore, such cells are used as immunizing agent in the above described protocol.

The important characteristics of the preferred monoclonal antibodies for the immunotoxins are (1) their immunoglobulin class, (2) their selectivity for human breast and/or ovarian cancer cells, (3) the range of human breast cancer cell lines to which they bind, (4) the range of human breast tumor frozen sections to which they bind, and (5) their ability to form an active immunotoxin.

The selectivity and range of a given preferred antibody for the immunotoxin is determined by testing it against panels of (1) human breast cancer tissues and cells and (2) normal human tissues or cells of breast or other origin. In selecting the preferred class of antibodies, approximately 22,000 growing hybridoma cultures were initially screened against the immunizing breast tumor membranes or cell line, a panel of seven normal tissue membranes, a fibroblast cell line, and a breast tumor frozen section. Clones that reacted with the neoplastic materials, but not with the normal materials, were identified in this initial screen and chosen for isotyping and additional screening for selectivity and range. The additional screening involved: sixteen normal tissue sections, five normal blood cell types, eleven non-breast neoplasm sections, twenty-one breast cancer sections, and fourteen breast cancer cell lines.

For the preferred antibodies for the immunotoxins, the words "specificity" and "normal tissue reactivity" are used interchangeably and are defined as the sum of the number of substructures stained in sixteen normal tissue frozen sections and the number of blood cell types bound, divided by the sum of the total number of substructures bound by any of the monoclonal antibodies in all the tissue on which the monoclonal antibodies were tested and five blood cell types tested.

The term "tumor range" is defined as the number of breast tumor frozen sections stained divided by the number of breast tumor frozen sections tested. The term breast cancer "cell line range" is defined as the number of breast cancer cell lines stained divided by the number of breast cancer cell lines tested. The antibodies of the immunotoxins herein preferably have a normal tissue reactivity equal to or less than 0.09, and a breast tumor binding range of equal to or greater than 0.25 or a breast cancer cell line binding range of equal to or greater than 0.25.

Antibodies of five of the thirty-three deposited hybridomas herein were found to recognize the same 200K dalton antigen. Antibodies of four of the thirty-three bound to a 240K dalton intracellular antigen. Three bound to one or more high molecular weight mucins (HMW) and two bound to transferrin receptors in the form of a 95K dalton antigen. All antigen weights mentioned herein were determined by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis under reducing conditions using procedures known in the art.

The immunotoxins herein may be prepared by conjugating a toxin as described above, such as ricin A chain, to one of the above-described antibodies using the coupling agents defined above. The technique for preparing such immunotoxins is described in EP Publication No. 153,114, published Feb. 8, 1985, the disclosure of which is incorporated herein by reference.

The following examples provide a detailed description of the preparation and characterization of representative monoclonal antibodies for conjugation as well as an immunotoxin for use with IL-2 in accordance with this invention. These examples are not intended to limit the invention in any manner. In the examples, all parts and percentages for solids are given by weight/weight unless otherwise indicated, and all parts and percentages for liquids are given by volume/volume unless otherwise indicated.

EXAMPLE I

Antibody Characterization

Fresh post-surgical human breast cancer tissue and a variety of normal tissues were used to prepare membrane extracts by homogenization and discontinuous sucrose gradient centrifugation. Human breast cancer cell lines were obtained from the Breast Cancer Task Force, from the American Type Culture Collection (ATCC), and from Dr. Jorgen Fogh at Memorial Sloan Kettering. The cells were maintained and passaged as recommended by the Breast Cancer Task Force, the ATCC, and Dr. Fogh. For immunizations, either membrane extract containing 100 μg of protein (Lowry assay) or ten million live breast cancer cells were inoculated intraperitoneally into five-week-old Balb/c mice. The mice were boosted identically twice at monthly intervals. Three days after the last boost, the spleens were removed for cell fusion.

Hybridoma Methods

Somatic cell hybrids were prepared by the method of Buck, D. W., et al, supra, using the murine myeloma line Sp-2/0/Ag14. All hybridoma cell lines were cloned by limiting dilution. Half of the fusions employed splenocytes from mice immunized with breast cancer membrane extracts and half used splenocytes from mice immunized with live breast cancer cell lines. Eighty-three thousand four hundred twenty-four wells were generated from those fusions, of which 22,459 exhibited hybridoma growth.

Screening Methods

Hybridoma supernatant was assayed for reactive antibody in either a solid phase enzyme-linked immunosorbent assay (ELISA) with the immunizing breast cancer membrane extract or an indirect immunofluorescence assay with the immunizing breast cancer cell line. For the solid phase membrane ELISA, 40 μl of 0.1 mg/ml breast cancer membrane protein were placed in polyvinyl chloride (PVC) microtiter wells for 12 hours at 4° C. The extract was aspirated and the wells were washed with phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA). The wells were then incubated with 45 μl of a 1:10 dilution of hybridoma supernatant. The diluent was medium with 25 mM of a buffer, 10% bovine serum, and 0.1% sodium azide. After 30 minutes at room temperature, the wells were again washed and incubated 45 minutes at 37° C. with a 1:200 dilution of peroxidase conjugated goat anti-mouse IgG. The diluent was PBS. The wells were then washed with PBS and reacted with 200 μl of 1,2-azino-di(3-ethylbenzthiazoline sulphonic acid) in 0.1M sodium citrate buffer pH 4.2 for 30 minutes at room temperature. Optical density was measured at 405 nm on a MicroElisa Reader. For each experiment a positive control, anti-beta 2 microglobulin at 5 μg/ml, was reacted with normal human kidney membrane. This gave an optical density of 1.0±0.1 (standard deviation). The background was 0±0.1 optical density units (O.D.) using medium without mouse monoclonal antibody. Wells that gave a reaction on the breast cancer membrane extract of greater than 0.7 O.D. were saved.

For the indirect immunofluorescence cell line assay 100,000 breast cancer cells of the immunizing cell line were placed overnight with appropriate media in each chamber of a set of eight chambered slides. Similarly, 100,000 fibroblast cells from cell line CC95 were incubated overnight in chambered slide wells. The cells were washed with PBS containing 1% BSA. The wells, both breast cancer and fibroblast, were incubated for 30 minutes at 4° C. with 1:10 dilutions of hybridoma supernatant. The cells were again washed and incubated 30 minutes at 4° C. with a 1:50 dilution of fluorescein isothiocyanate (FITC)-conjugated goat $F(ab')_2$ anti-mouse Ig. The cells were washed three times, fixed in 1.5% formaldehyde in PBS for five minutes, and the chambers removed and rinsed in PBS. The slides were then mounted in a composition containing polyvinyl alcohol, glycerol, buffers and a preservative and examined with a fluorescence microscope. Hybridoma wells showing strong fluorescent binding to the breast cancer cells but no fluorescent binding to fibroblasts were saved. Five thousand one hundred fifty-six hybridoma wells revealed breast cancer reactivity in the initial screen.

Supernatants from the 5156 positive wells were then tested in solid phase ELISA with seven normal tissue membrane extracts (liver, lung, colon, stomach, kidney, tonsil, and spleen). Any well supernatant giving an ELISA O.D. greater than 0.3 was discarded. One thousand one hundred one of the supernatants were found to be unreactive with the normal tissue extracts.

The 1101 hybridoma supernatants were tested on frozen sections of human breast carcinoma tissues. Six micron sections were attached to slides, fixed 10 minutes in acetone at 4° C., dried 10 minutes at room temperature, washed with PBS, blocked with horse serum and incubated 20 minutes at room temperature with 100–200 μl neat hybridoma supernatant. The slides were washed with PBS, and finally incubated 20 minutes at 37° C. with a 1:50 dilution of peroxidase conjugated rabbit anti-mouse Ig, washed again with PBS, and finally incubated 7.5 minutes at 37° C. with 0.5 mg/ml diaminobenzidine in 0.05M Tris buffer pH 7.2 containing 0.01% hydrogen peroxide. The slides were stained with hematoxylin, dehydrated and mounted in a medium containing 35.9% methyl/n-butylmethacrylate copolymer, 7.1% butyl benzyl phthalate, and 0.3% 2,6-ditertbutyl-p-cresol. One hundred twenty-four wells yielded breast cancer selective binding and were cloned.

Purification and Class Determination

Immunoglobulin class and subclass of the monoclonal breast cancer selective antibodies were determined by an immunodot assay essentially the same as that described in McDougal et al., *J. Immunol. Meth.* 63:281–290 (1983). Antibodies were also internally labeled by growing $2–3\times10^6$ hybridoma cells for four hours in methionine-free medium containing 0.2 μCi ^{35}S methionine. ^{35}S-labeled antibodies were immunoprecipitated with fixed staphylococcus A cells, or with fixed staphylococcus A cells precoated with rabbit anti-mouse immunoglobulin, and the immunoprecipitates were analyzed by SDS-PAGE to determine antibody light and heavy chain mobility, lack of extra chains, and the ability of each antibody to bind staphylococcal protein A.

The antibodies were expanded in vivo. Balb/c or F1 (C57B/6×Balb/c) mice were primed with 0.5 ml pristane intraperitoneally (ip) and after 10–14 days inoculated with one million log phase hybridoma cells in PBS. Ascites fluid was stored at −70° C. and thawed and filtered through a 0.8 micron filter unit before further purification.

Some IgG antibodies that bound staphylococcal protein A were purified by affinity chromatography on protein A-chromatographic resin containing either agarose, dextran and/or acrylamide with pH step gradient elution. IgG antibodies that did not bind protein A were precipitated by addition of ammonium sulfate to 40% saturation at 0° C. or by binding to DEAE or Affigel TM (Biorad, Richmond, Calif.). Alternatively, IgG antibodies were purified by chromatography using a Sephacryl S-200 column, followed by DEAE cellulose.

The precipitates were redissolved in PBS, dialysed to 20 mM Tris pH 7.2 and chromatographed on a 1.6×50 cm column of diethylaminoethyl cellulose (DEAE) eluting with a 1.5 liter 0–600 mM NaCl gradient at 4° C. at a flow rate of 1 ml/min. In each case, column fractions were monitored by SDS-PAGE and the purest antibody fractions were pooled, concentrated to 1–3 mg/ml, dialysed to PBS/0.02% NaN_3, and stored at 4° C.

IgM antibodies were purified by gel filtration material on a 2.6×40 cm column of Sephacryl S-300 or other gel filtration or resin containing agarose, dextran and/or acrylamide, eluting with PBS/0.01% sodium azide at room temperature at a flow rate of 1 ml/min.

Selectivity Determination

For evaluation of their selectivity for breast cancer, the purified antibodies were tested by immunoperoxidase section staining on sections of sixteen normal tissues, and by immunofluorescent cell sorting on five blood cell types. Immunoperoxidase staining was performed as above except that known dilutions of purified antibodies in PBS in the range of 1–40 μg/ml were used instead of hybridoma supernatants. The pure antibodies were first titrated to find the minimal concentration

giving strong immunoperoxidase staining on breast cancer sections and then used at the concentration for the normal tissue tests. Peripheral bood cells (platelets, lymphocytes, red blood cells, granulocytes, and monocytes) were prepared by centrifugation using a medium which separates monocytes from polymorphonuclear leukocytes. The cells were reacted with antibody at the optimal concentration determined above for 30 minutes at 4° C., washed, reacted with a 1:50 dilution of fluorescein isothiocyanateconjugated goat anti-mouse Ig for 30 minutes at 4° C., washed again, and examined in a cell sorter. The wash buffer and diluents were PBS with 1% gelatin and 0.02% sodium azide. The cell sorter was equipped with a 76 micron nozzle and a one watt argon ion laser at 488 nm. An 80 mm confocal lens was used on the optical rail assembly for focusing. Other filters used were a 515 nm interference filter and a 515 nm absorbance filter (for scattered laser light) and a neutral density 1.5 filter for forward angle light scatter. Contour plots of log fluorescein fluorescence versus forward angle light scatter were used for sample analysis. No blood cell types showed detectable binding.

The binding behaviors of the preferred antibodies of the second class herein are reported in Table I below. The following abbreviations are used to denote structures bound by the antibodies: Ac, acini; G, glands; T, tubules; D, ducts; L, lumen; W, sweat glands; E, epithelium; S, sebaceous glands; Gr, granulocytes; Mk, megakaryocytes; M, macrophage; Ly, lymphocytes; Bl, Basal layer; Fe, focal epithelium; A, alveolar lining cells; B, Bowman's capsule; Mu, muscle; and I, islets; H, hair follicles; U, glomeruli; and V, vessels/endothelial.

TABLE I

	Normal Tissue Bindings of MABS															
MAB	Pan-creas	Eso-phagus	Lung	Kidney	Colon	Sto-mach	Brain	Tonsil	Liver	Heart	Ovary	Skin	Bone Marrow	Uterus	Bladder	Normal Breast
2G3	2Ac	2E	1A	2T	P	1L	0	1E	0	0	0	0	0	2L	2E	2E
9C6	0	2E	0	0	0	1L	0	1Ly, 2E	0	0	0	0	2Gr	0	0	2E
32A1	1D	1E	1A, M	1T, U	0	0	1My	0	0	0	0	1S, W	0	1Mu	0	0
33F8	0	2E	0	1T	0	0	0	1Ly	0	0	0	1W	1Mk	1L	1E	0
35E10	0	2E	1A, 1M	0	0	1G	0	1Ly	0	0	0	2W	2Gr	0	0	0
41B4	0	0	0	0	0	0	0	1E	0	0	0	1W	0	0	0	1E
87H7	1D	1E	0	0	1G	1G	0	1Ly, E	2	0	0	2H	0	0	0	1E
106A10	1Ac, D	1E	1M	0	0	0	0	2E	2D	0	0	2E, W	0	1G	2E	2E
113F1	2Ac	2E	0	0	0	2G	0	1E	0	0	0	0	0	0	1E	0
120H7	0	1E	0	1T	0	1L	0	0	0	0	0	2S	0	2L	0	0
140A7	1Ac, D	0	0	2T, B	0	0	1Ly	2	0	0	2E, 1S	0	0	0	0	0
200F9	1Ac	0	0	2L	0	0	0	0	0	0	0	2S	0	0	0	0
203E2	2Ac	1E	2A	2L	0	0	0	0	0	0	0	2S				0
219F3	1Ac	2E	0	1T	0	0	0	1Ly, E	0	0	0	2H, W	1-2Gr	1G	0	2E
245E7	1L	0	1A, M	0	0	2L	0	1E	0	0	0	2S	0	2L	1E	2L
254H9	2Ac	2E	1A	2T	0	1G	0	0	0	0	0	0				2L
260F9	1Ac	2E	0	1T	0	1G	0	2E	2D	0	0	2E, 2H	0	1L	2E	2E
266B2	1Ac, 1D	2E	0	1T	0	0	0	2E	0	0	0	2E, 2W	0	0	1E	1E
317G5	1Ac, 1	0	0	2T	1G	0	0	0	2D	0	0	0	0	1G	0	0
369F10	0	0	0	0	0	1G	0	0	0	0	0	1S	0	0	0	0
387H9	1D	0	0	0	0	0	0	1Ly, 1E	1, 1D	0	1V	0	2	1G	0	1
421E8	1Ac	1E	0	1T	0	1G	0	0	1	0	0	0	0	1G	0	0
451C3	0	0	2M	0	0	0	1V	2Ly, 1Bl	0	0	0	0	2	1G	0	0
452E12	0	0	0	0	0	0	0	0	1	0	0	2S	0	0	0	2
452F2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
454A12	0	0	1M	0	1G	0	0	0	0	0	0	1E, H	0	1G	1E	1E
454C11	1D	1-2E	0	1T	0	0	0	1E	1D	0	0	1E, H	0	1G	1E	1E
457D7	0	0	0	0	0	1G	0	0	1	0	0	2S	0	0	0	2
520C9	0	0	0	1T	0	0	0	0	0	0	0	0	0	0	0	0
650E2	1Ac, 1	0	1-2A	2T	2G	0	0	0	2D	0	0	0	0	2G	0	1
697B3	0	0	0	2T	0	0	0	0	0	0	0	2S	0	2L	0	2L
741F8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
759E3	0	0	0	0	0	0	0	1E	0	0	0	0	0	0	0	0
788G6	0	0	0	2T	0	0	0	1Fe	0	0	0	0	0	0	0	0

0 = No binding
1 = Moderate binding
2 = Strong binding

Breast Cancer Cell Binding Range Determination

Antibodies were further evaluated for range of breast cancer cell line recognition by immunofluorescence assays on 14 breast cancer cell lines. Table II below reports the results of these tests for the preferred antibodies herein of the second class.

Non-Breast Cancer Binding of Monoclonal Antibodies

Finally, the antibodies were tested by immunoperoxidase staining on eleven non-breast malignancies. The results for the preferred antibodies herein for the second class are reported in Table III below. The numbers are the same as designated in Table 1.

TABLE II

	Breast Cancer Cell Line Binding of MABS													
MAB	MCF7	BT20	ZR751	MDA-MB231	CAMA1	ALAB	BT549	BT474	T47D	MDA-MB157	MB330	MDA-SKBR3	MDA-BT483	ZR7530
2G3	4	3	3	2	4	2	4	2	3	2		3	4	3
9C6	3	0	3	0	4	2	0	3	3	0	0	2	0	2
32A1	3	2	2	2	2	2	0	3	3	2	0	2	1	3
33F8	2	3	2	0	2	3	2	2	0	3	2	2	0	1
35E10	1	0	0	0	0	0	0	2	0	0	0	0	0	2
41B4	1	0	0	0	0	0	0	0	1	0	0	0	0	0
87H7	0	0	1	1	0	0	0		0	0	0	1	0	0
106A10	3	3	2	2	2	0	0	2	3	2	2	3	2	2
113F1	3	4	2	2	4	0	0	4	3	3	2	4	2	0
120H7	3	2	3	0	3	0	2	0	0	0	0	2	3	2
140A7	3	2	1	0	2	0	1	0	0	0	3	0	2	1
200F9	3	3	2	0	2	2	3	0	3	2	0	0	3	2
203E2	4	4	3	0	4	2	4	2	4	3	0	2	4	3
219F3	3	3	4	0	4	3	2	3	3	4	0	3	2	3
245E7	4	4	4	2	4	2	4	2	4	4		3	4	4
254H9	4	4	4	2	4	1	3	2	4	4		2	3	3
260F9	3	3	3	2	3	2	0	2	2	2	ND	4	2	3
266B2	3	2	2	2	3	0	0		2	2		2	2	2
317G5	2	3	3	0	4	3	1		3	4	0	3	2	3
369F10	1	0	0	0	2	0	0		0	0		0	2	0
387H9	3	2	2		3	3	0		2	2		2	2	2
421E8	2		2		2		0					0	2	0
451C3	3	2	2		2	2	2		4	2		2	0	2
452E12	0	0	0		0	0	0		0	0		0	0	0
452F2	0	1	2		2	2	0		1	0		3	2	3
454A12	2	2	2		2	3	2		3	2		2	2	2
454C11	2	2	2	2	2	2	1		1	0		4	2	4
457D7	0	0	0		1		0		0	0		0	2	0
520C9	1	0	1		2		0		1	0		3	2	2
650E2	3	2	3		3		0		3	3		3	2	3
697B3	2				4		3		4	3		0	4	2
741F8	1				2		0		2	0		4	2	2
759E3	0				2				2			3	0	4
788G6	2				2				3			0	3	2

0 = Negative
1 = Weak
2 = Moderate
3 = Strong
4 = Very strong

TABLE III

	Nonbreast Cancer Bindings of MABs										
MAB	Colon	Lung	Prostate	Pancreas	Uterus	Lymphoma	Stomach	Bladder	Esophagus	Melanoma	Ovarian
2G3	2	0	2	0	2	0	2	0	0	0	2
9C6	1	0	0	0	2	1	0	0	0	0	1
32A1	0	0	0	2	0	0	1	0	0	0	0
33F8	0	1	0	0	1	0	0	0	0	0	1
35E10	2	2	1	2	0	2	0	0	0	0	0
41B4	0	0	2	0	0	0	0	0	0	0	0
87H7	0	1	2	0	1	0	0	2	1	0	0
106A10	0	1	1	0	1	1	1	0	1	0	0
113F1	0	2	0	2	1	2	2	0	1	0	0
120H7	0	0	2	0	1	1	0	0	0	0	2
140A7	0	0	0	1	2	1	2	1	0	0	0
200F9	0	1	0	0	0	0	0	0	0	0	1
203E2											
219F3	0	0	1	0	1	1	0	0	0	0	1
245E7	0	2	2	2	2	2	0	0	0	0	2
254H9											
260F9	0	0	1	1	1	0	0	0	1	0	2
266B2	0	1	1	1	1	0	1	0	1	0	1
317G5	1	1	0	0	1	0	0	0	0	0	1
369F10	0	1	1	1	0	0	0	0	0	0	2
387H9											
421E8	1	1	1	1	1	0	0	0	1	1	1
451C3	1	1	1	1	2	1	2	1	2	1	1
452E12	0	0	1	0	1	0	0	0	1	0	0

TABLE III-continued

	Nonbreast Cancer Bindings of MABs										
MAB	Colon	Lung	Pro-state	Pan-creas	Uterus	Lym-phoma	Sto-mach	Bladder	Eso-phagus	Melanoma	Ovarian
452F2	0	0	0	0	0	0	0	0	0	0	0
454A12	0	0	0	1	2	0	1	1	2	2	1
454C11	0	0	1	1	2	0	0	0	0	0	1
457D7	0	0	1	0	0	0	0	0	0	0	0
520C9	0	1	1	1	1	0	0	0	0	0	0
650E2	2	2	2	2	2	0	0	0	0	0	2
697B3	0	0	0	0	0	0	0	0	0	0	0
741F8	0	0	0	0	0	0	0	0	0	0	0
759E3	0	1	1	1	0	0	0	0	0	0	0
788G6	0	2	0	0	0	0	0	0	0	0	2

The tumor breast cancer range, breast cancer cell binding range, and blood cell binding and selectively characteristics for the monoclonal antibodies according to the invention are summarized in Table IV.

TABLE IV

	MAB Candidates			
MAB	Blood Cells	Tumor Range	Cell Range	Selectivity
2G3	0	1.00	1.00	0.078
9C6	0	0.86	0.57	0.063
32A1	0	0.33	0.79	0.078
33F8	0	0.19	0.71	0.063
35E10	0	0.62	0.14	0.070
41B4	0	0.67	0.00	0.023
87H7	0	0.95	0.00	0.078
106A10	0	0.86	0.86	0.086
113F1	0	0.14	0.79	0.047
120H7	0	0.67	0.57	0.047
140A7	0	0.71	0.36	0.070
200F9	0	0.52	0.71	0.031
203E2	0		0.86	0.055
219F3	0	0.86	0.86	0.086
245E7	0	1.00	1.00	0.070
254H9	0		0.92	0.064
260F9	0	0.52	0.92	0.089
266B2	0	0.71	0.83	0.070
317G5	0	0.43	0.77	0.055
369F10	0	0.81	0.17	0.023
387H9	0	0.29	0.91	0.086
421E8	0	0.81	0.57	0.055
451C3	0	0.38	0.91	0.070
452E12	0	0.52	0.00	0.023
452F2	0	0.24	0.55	0.000
454A12	0	0.29	1.00	0.031
454C11	0	0.76	0.75	0.078
457D7	0	0.55	0.10	0.039
520C9	0	0.25	0.40	0.008
650E2	0	0.86	0.90	0.008
697B3	0	0.81	0.88	0.070
741F8	0	0.18	0.63	0.000
759E3	0	0.14	0.78	0.008
788G6	0	0.62	0.83	0.016

Antibody Affinity and Antigen Density

Several of the antibodies which may be employed herein were iodinated and tested for binding to MCF-7, CAMA1, SKBR3, or ZR7530 cells. The antibodies were labeled with ^{125}I using chloramine T to a specific activity of approximately 10 $\mu Ci/\mu g$. To determine immunoradiochemical purity, 100,000 cpm of two of the labeled antibodies in 0.5 ml fetal calf serum was serially absorbed with five aliquots of target cells for 15 minutes at 0° C. (generally 4,000,000 cells per aliquot), and the remaining radioactivity in the supernatant after each absorption was determined.

For measurements of association constants, known concentrations of labeled and unlabeled monoclonal antibodies were incubated with target cells in fetal calf serum for 15 minutes in ice. Aliquots of the cell/antibody mix were then counted in a gamma counter or filtered through Microfold filter plates (V & P Scientific) and the filters counted. To account for unbound antibody retained in liquid on the filters, controls containing the same concentrations of antibody, but no cells, were done in parallel. Association constants and antigen copy number per target are calculated from the affinity test results and are reported in Table V below.

TABLE V

	Affinity and Antigen Copy Number of MABs		
MAB	n	Ka	Cell Line
2G3	3700000	9.1×10^6	MCF7
9C6			
32A1			
33F8			
35E10			
41B4			
87H7			
106A10			
113F1	2300000	1.1×10^9	MCF7
120H7	210000	6.2×10^6	MCF7
140A7			
200F9			
203E2			
219F3			
245E7			
254H9			
260F9	30000	6.0×10^7	MCF7
266B2	80000	2.7×10^8	MCF7
317G5	3200000	1.6×10^6	CAMA1
369F10			
387H9			
421E8			
451C3	400000	1.4×10^8	MCF7
452E12			
452F2	250000	6.8×10^6	SKBR3
454A12	470000	1.2×10^8	MCF7
454C11	390000	4.8×10^7	ZR7530
457D7			
520C9	500000	8.2×10^6	SKBR3
650E2			
697B3			
741F8			
759E3			
788G6			

Characterization of Antigen Specificity

For identifying the antigens recognized by the monoclonal antibodies, immunoprecipitation of the antigens was carried out according to the following method. Eight mm diameter polystyrene balls (Precision Plastic Ball Co.) were covered with 10% fuming nitric acid in glacial acetic acid and were incubated for three hours in a 50° C. water bath. Following the acid treatment, the balls were rinsed three times with distilled water, covered with 1% sodium dithionite in 0.1M NaOH and incubated three hours in a 50° C. water bath. The balls were again rinsed three times with distilled water, cov-

ered with 0.1% 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDAC), 0.2% suberic acid (suberic acid dissolved in dimethylformamide) and incubated overnight at room temperature. The balls were rinsed three times with distilled water, and marked for identification.

Purified monoclonal antibodies were diluted 0.2 mg/ml in 2-(N-morpholino) ethane sulfonic acid buffer, and the previously treated and marked polystyrene balls were placed in individual tubes and covered with 450 microliters diluted antibody and 50 microliters of fresh 1% EDAC. Tubes were capped and incubated at 25° C. for 24 hours. Following this incubation, the balls were rinsed twice with PBS and were either used fresh or were stored for several days at 4° C. before use.

Freshly labeled target cell extracts were prepared from human breast cancer cell lines labeled with ^{125}-I by the lactoperoxidase method of Marchalonis, J., "An Enzymic Method for the Trace Iodination of Immunoglobulins and Other Proteins", *Biochem. J.* 113:299–305 (1969), or with 35-S by growth in 35-S methionine. The labeled cells were dissolved in solubilization buffer (1% (v/v) Triton X-100, 150 mM NaCl, 5 mM EDTA, 25 mM Tris-HCl, pH 7.5). Four parts of labeled extract were mixed in a vessel with one part solubilization buffer containing 50 mg/ml bovine serum albumin, to give a final concentration of 10 mg/ml BSA. The balls coated with monoclonal antibody were added to the vessel and were incubated four hours on ice with shaking. Labeled antigen was pipetted from the vessel and the balls were rinsed four times with solubilization buffer. The balls were then removed, placed in individual tubes with 100 microliter Laemmli SDS gel sample buffer, and were incubated three minutes in boiling water. The balls were removed and the samples were run on an SDS gel with appropriate standards.

Immunoprecipitation tests on the antibodies indicated that five of them (454C11, 452F2, 520C9, 741F8, and 759E3) all bind a monomeric protein of about 200 K daltons found in cancerous breast tissue. Two of the five (520C9 and 741F8) are believed to recognize the same epitope on the 200K dalton protein. 454C11 and 759E3 bind a second epitope on the same antigen, and 452F2 binds a third epitope on the same antigen. Four of the antibodies (41B4, 87H7, 452E12, 457D7) bound to a 240K-daltons intracellular antigen. Seven antibodies (2G3, 200F9, 203E2, 245E7, 369F10, 697B3 and 788G6) bound to high molecular weight mucins (HMW). Two antibodies (451C3 and 454A12) bound to transferrin receptors in the form of a 95,000-daltons dalton antigen. Neither 451C3 nor 454A12 blocked binding of transferrin to the receptor. The antigen binding characteristics of the monoclonal antibodies according to the invention are summarized in Table VI.

TABLE VI

MAB	Antigen
2G3	HMW Mucin
9C6	70K
32A1	
33F8	66K
35E10	80K
41B4	240K
87H7	240K
106A10	55K a
113F1	40, 60, 100, 200K Very Diffuse
120H7	HMW Mucin
140A7	Glycolipid (pentasaccharide)
200F9	HMW Mucin
203E2	HMW Mucin
219F3	
245E7	HMW Mucin
254H9	
260F9	55K b
266B2	55K b
317G5	42K c
369F10	HMW Mucin
387H9	40K
421E8	
451C3	Transferrin receptor
452E12	240K
452F2	200K
454A12	Transferrin receptor
454C11	200K
457D7	240K
520C9	200K
650E2	42K c
697B3	200K
759E3	200K
788G6	HMW Mucin

a = Different epitope than that bound by 260F9 and 266B2
b = Different epitope than that bound by 106A10; both 260F9 and 266B2 appear to bind to same epitope
c = Cross block each other

Antibody Isotype

Antibody isotype was determined as follows: A grid of 5-mm squares was lightly drawn in pencil on the nitrocellulose sheet and 1-ml droplets of antiisotype sera (Litton Bionetics, Kensington, Md., rabbit antisera to mouse κ, λ, α, γ1, γ2a, γ2b, γ3, and μ chains) were applied so that each row of squares received one spot of each heavy and light chain reagent. The sheet was incubated one hour at room temperature in a moist chamber, rinsed quickly in PBS-BSA, containing 1% (w/v), and left overnight in PBS-BSA at 4° C. Strips were cut apart with a scissors and stored at 4° C. in PBS-BSA containing 0.02% sodium azide. Alternatively, strips were air-dried and stored desiccated at 4° C. A series of small tubes was prepared containing 3 ml hybridoma culture supernatant or supernatant diluted with PBSBSA. 1:10 dilutions were generally successful; and some supernatants can be diluted as much as 1:200. A nitrocellulose strip was incubated in each tube for one hour at room temperature. The strips were rinsed three times in PBS-BSA and incubated for one hour at room temperature in diluted rabbit anti-mouse-horseradish peroxidase. The strips were rinsed twice in PBS-BSA and twice in Tris buffer. The strips were placed in Tris buffer containing diaminobenzidine and hydrogen peroxide until sufficient color developed on the anti-isotype spots (usually 3–4 minutes). The antibody isotypes are indicated in Table VII.

TABLE VII

	Isotype of MABs
MAB	Isotype
2G3	G1
9C6	M
32A1	G1
33F8	G1
35E10	M
41B4	G1
87H7	G1
106A10	G1
113F1	G3
120H7	M
140A7	M
200F9	G1
203E2	G1
219F3	G1

TABLE VII-continued

Isotype of MABs	
MAB	Isotype
245E7	G1
254H9	M
260F9	G1
266B2	G1
317G5	G1
369F10	M
387H9	G1
421E8	G1
451C3	G1
452E12	G1
452F2	G1
454A12	G1
454C11	G2A
457D7	G1
520C9	G1
650E2	G1
697B3	G1
741F8	G1
759E3	G1
788G6	G1

EXAMPLE II

A. Preparation of Ricin A Chain

A soluble recombinant ricin A which requires no solubilization to be subjected to purification and to display cytotoxicity was prepared in accordance with the procedure described in copending U.S. application Ser. No. 837,583, filed Mar. 7, 1986, the disclosure of which is incorporated herein by reference. Briefly, when the coding sequence for ricin A was placed in direct reading frame with the DNA encoding leader sequence of phoA to form a putative fusion peptide, so that the leader sequence is the N-terminal portion of a leader/ricin A chimera, the ricin A sequences so disposed result in the soluble cytotoxic material.

Expression vectors containing the genes for the precursor proteins contained in pRT3 (ATCC Deposit No. 67,027, deposited Mar. 7, 1986), pRT17 (ATCC Deposit No. 67,026, deposited Mar. 7, 1986), and pRT38 (ATCC Deposit No. 67,025, deposited Mar. 7, 1986) or their mutagenized forms were constructed. Transforming host cells with these expression vectors resulted in solubilization of the precursor protein encoded. The arg-arg modified precursor was cleaved with trypsin; the A and B portions of the precursors were produced as separate proteins, as herein described.

In the phoA expression system, the essential component is the terminated phoA leader sequence upstream of, proximal to, and out of frame with the ricin A encoding sequence, wherein the ricin A encoding sequence is initiated by an ATG codon. The two coding sequences must be, of course, provided with a compatible bacterial promoter, which was the phoA promoter already associated with the leader. Additionally, production was improved in the presence of a positive retroregulator sequence which was the positive retroregulator sequences associated with the crystal protein of *B. thuringiensis*, which are described extensively in European Publication 174,785 published Mar. 19, 1986 and incorporated herein by reference. This was provided on bacterial transport vectors which included replicons and selectable markers.

The vectors were then used to transform a suitable procaryotic host, which was grown under conditions suitable for the particular host chosen, most frequently under conditions whereby the promoter placed in control of the expression system was suppressed. The production of the ricin A was then induced by providing conditions which effect expression under control of the chosen promoter and the production permitted to proceed for sufficient time to effect a desired accumulation of product. The protein product was then isolated by disrupting the cells and the cellular debris was removed. The ricin A produced was then further purified using standard techniques known in the art as applied to freely soluble proteins. However, the efficiency of the extraction and purification was enhanced by treating partially clarified extract with phenyl sepharose. The solubility of the ricin A in the sonicate (once separated from the membrane or other associated materials) was shown by its ability to remain in the supernatant when the sonicate was subjected to centrifugation at high speed, 100,000×g for 30 minutes, to spin down insoluble proteins.

A total of 2 ml of this soluble ricin A (at 9.0 mg/ml) was reduced by adding 2 μl of fresh β-mercaptoethanol (to 0.1%) and incubating at room temperature overnight. The 2 ml of reduced ricin A was applied to a G-25 column (Pharmacia) equilibrated with 0.10M sodium phosphate pH 8.0, followed by 0.5 ml of buffer to make 2.5 ml sample application volume. The next 3.0 ml of eluate (buffer was applied) was collected as desalted ricin A.

B. Conjugation of Ricin A to Antibody

A cell line producing an anti-breast monoclonal antibody designated 520C9, described more fully above, was deposited as Accession No. HB8696 on Jan. 8, 1985 in the American Type Culture Collection (ATCC), Rockville, Md. This antibody was reacted with 5,5'-dithiobis-(2-nitrobenzoic acid) at room temperature and then chilled, and then sufficient 2-iminothiolane (IT) was added to give 2.5 IT molecules per antibody molecule, as described in copending U.S. application Ser. No. 842,476, filed Mar. 21, 1986, the disclosure of which is incorporated herein by reference.

A total of 166 μl of propylene glycol was added to 0.84 ml of the IT-derivatized antibody. The 2.32 ml of ricin A chain described above was added to initiate the conjugation reaction. The mixture was incubated at room temperature for two hours.

The conjugation reaction mixture was applied to a Zorbax-GF-250 sizing (gel filtration) HPLC column using an eluting buffer of 0.15M sodium phosphate, pH 8.0. A total of 78% recovery of the purified immunoconjugate was obtained from the column.

C. IL-2

The recombinant IL-2 employed in this example is des-ala$_1$-IL-2$_{ser125}$. The amino acid sequence of this IL-2 differs from the amino acid sequence of native human IL-2 in that it lacks the initial alanine of the native molecule, and the cysteine at position 125 has been changed to serine. Samples of *E. coli* that produce this IL-2 have been deposited by Cetus Corporation in the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Md., USA on Sept. 26, 1983 under accession number 39,452 and on Mar. 6, 1984 under accession number 39,626 under the provisions of the Budapest Treaty.

The IL-2 was processed and purified as described in the text and FIG. 1 of U.S. Pat. No. 4,604,377 issued Aug. 5, 1986, the disclosure of which is incorporated herein by reference, except that the in vitro disulfide bond formation was carried out using cupric chloride, as described in U.S. Pat. No. 4,572,798 rather than o-iodosobenzoate. When the IL-2 was recovered from the

chromatography step(s) it was lyophilized and resuspended in a neutral aqueous buffer. The purity of the recombinant IL-2 after the chromatography step(s) was at least about 95% and the IL-2 contained less than about 0.02 ng/ml endotoxin as determined by the Limulus amebocyte assay.

The purified IL-2 was formulated at a concentration of 0.3 mg/ml with 50 mg/ml mannitol.

D. Model

The target cells employed are murine tumor P388 leukemia cells, obtainable from the American Type Culture Collection, Rockville, Md.

E. Subcutaneous Tumor Injection

The tumor cells are harvested from culture suspensions inoculated subcutaneously (sq) or intraperitoneally (ip) into the appropriate type of mouse.

F. Results

IL-2 alone, the immunotoxin described above alone, and IL-2 with the immunotoxin may be administered intraperitoneally to the mice, beginning one day after tumor implantation (Day 1), with the dose and schedule indicated in Table VIII.

TABLE VIII

Agent(s)	Dose/kg	Schedule
PBS		
IL-2 alone	3.75–7.5 × 10^6 U/kg	Daily for 14 days-begin at Day 1 post-implant
Immunotoxin alone	100 μg/kg	Daily for 7 days-begin at Day 1
IL-2 and Immunotoxin	3.75–7.5 × 10^6 U/kg 100 μg/kg	Daily for 14 days-begin at Day 1 Daily for 7 days-begin at Day 1

The maximum tolerated dose of IL-2 was found to be between 50 and 100 KU Df IL-2 given daily to nude mice for 14 days and 150–200 KU of IL-2 given daily to immunocompetent mice for 14 days. The administration of the combination of agents as provided in Table VIII is expected to reduce tumor growth greater than the administration of either agent alone.

In an alternative scheduling to that given in Table VIII, the maximum tolerated dose of IL-2 may be administered alone for one week daily ip or im near the tumor, followed by administering one-half the maximum tolerated dose of IL-2 and the maximum tolerated dose of the immunotoxin, as separate intravenous boluses. The dosage and scheduling must be adjusted to obtain efficacious results. Each type of cancer and immunotoxin will require different dosages and schedules, to be determined by routine experimentation.

EXAMPLE III

An immunotoxin (IMT) was constructed as described in Example II except that an anti-breast monoclonal antibody designated 260F9, described more fully above (deposited as ATCC No. HB-8488)was employed instead of 520C9. The resulting immunotoxin was diluted in saline and 0.01% mouse serum albumin.

The IL-2 employed was the same as that used in Example II. The target cells employed were human breast carcinoma cells from a cell line designated MX-1 obtained from the National Institutes of Health. The tumor cells were implanted sq into nude mice.

The scheduling and dosing were as follows. The immunotoxin was administered every other day for a total of six times intravenously at 3.5 μg/20 g mouse and 7.0 μg/20 g mouse. The IL-2 was administered daily for nine days intraperitoneally at 10 kilounits/dose and 100 kilounits/dose. Both started on day 0 (the seventh day after implantation of the tumor) and had overlapping schedules when administered concomitantly. The results are shown in Table IX below:

TABLE IX

Group	ΔBW*	Deaths	ΔTW**	% T/C***
IL-2				
10 Ku	1.03	0/5	15.0	76
100 Ku	1.14	1/5	9.3	47
IMT				
3.5 μg	0.98	0/5	9.4	47
7.0 μg	0.92	0/5	4.7	24
Combination				
10 KuIL-2/3.5 μg IMT	0.92	0/5	12.9	65
100 KuIL-2/3.5 μg IMT	0.95	1/5	11.3	57
10 KuIL-2/7.0 μg IMT	0.75	2/5	3.6	18
100 KuIL-2/7.0 μg IMT	0.86	2/5	0.9	4
Saline Control	1.09	0/5	19.8	100

*ΔBW is change in body weight as measured by the ratio of mean body weight (in g) at 14 days after treatment to mean body weight (in g) at the start of treatment.
**ΔTW is change in tumor volumes as measured by the ratio of mean tumor volume (in mm^3) at 14 days after treatment to mean tumor volume (in mm^3) at the start treatement.
***% T/C is the ratio of treated tumor volumes to control tumor volumes. (e.g., % T/C = 40 means there was 60% tumor growth inhibition)

The results indicate tat the combination was approximately additive with respect to its antitumor effects; toxicities were enhanced slightly. Alteration of the dose/route/schedule may alter the efficacy and toxicity results.

The monoclonal antibody-producing hybridomas listed below were deposited with the American Type Culture Collection (ATCC) or Invitro International Inc. (IVI) under the terms of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure and the Regulations thereunder (Budapest Treaty). This assures maintenance of the viable culture for 30 years from date of deposit. The hybridomas will be made available by ATCC or IVI under the terms of the Budapest Treaty, and subject to an agreement between the assignee of this application, Cetus Corporation, and ATCC or IVI which assures unrestricted availability upon issuance of the pertinent U.S. patent. Availability of the deposited strains is not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any government in accordance with its patent laws. The assignee has agreed that if the cell lines on deposit should die or be lost or destroyed when cultivated under suitable conditions, they will be promptly replaced on notification with a viable culture of the same cell line.

Each hybridoma designation listed in the left column of Table X corresponds to the monoclonal antibody producing the designated monoclonal antibody.

TABLE X

Cell Line Designation	IVI Accession Number
9C6	IVI-10056
41B4	IVI-10057
87H7	IVI-10059
106A10	IVI-10060
120H7	IVI-10061
200F9	IVI-10062
254H9	IVI-10063
421E8	IVI-10064
32A1	IVI-10066
35E10	IVI-10067
140A7	IVI-10069
203E2	IVI-10070

TABLE X-continued

219F3	IVI-10072
387H9	IVI-10073
452E12	IVI-10074
454A12	IVI-10075
457D7	IVI-10076
697B3	IVI-10077
741F8	IVI-10078
759E3	IVI-10079
788G6	IVI-10080
451C3	IVI-10081
452F2	IVI-10082
650E2	IVI-10083

Cell Line Designation	ATCC Deposit Date	ATCC Accession Number
260F9	1/27/84	HB-8488
2G3	1/27/84	HB-8491
33F8	1/9/85	HB-8697
113F1	1/27/84	HB-8490
245E7	1/27/84	HB-8489
266B2	1/27/84	HB-8486
317G5	1/27/84	HB-8485
369F10	12/13/84	HB-8682
454C11	1/27/84	HB-8484
280D11	1/27/84	HB-8487
520C9	1/8/85	HB-8696
*260F9-1C9	11/7/84	HB-8662

*This clone is a descendent of 260F9 and was found to be a better antibody producer than 260F9.

In summary, the present invention is seen to provide a combination therapy for cancer using an amount of anti-tumor immunotoxin and IL-2 together that is pharmacologically effective.

The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention. The present invention is not to be limited in scope by the cell lines deposited, since the deposited embodiment is intended as a single illustration of one aspect of the invention and any cell lines that are functionally equivalent are within the scope of this invention. The deposit of materials therein does not constitute an admission that the written description herein contained is inadequate to enable the practice of any aspect of the invention, including the best mode thereof, nor are the deposits to be construed as limiting the scope of the claims to the specific illustrations that they represent. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and fall within the scope of the appended claims.

What is claimed is:

1. A composition comprising a mixture, in pharmacologically effective amounts, of IL-2 from a mammalian species and at least one immunotoxin that binds selectively to cells containing the tumor burden.

2. The composition of claim 1 further comprising a pharmaceutically acceptable carrier medium for the IL-2 and immunotoxin.

3. The composition of claim 1 wherein the IL-2 is human IL-2.

4. The composition of claim 3 wherein the IL-2 is recombinantly produced.

5. The composition of claim 4 wherein the IL-2 is mature human IL-2, des-ala$_1$-IL-2$_{ser125}$, des-ala$_1$-IL-2$_{ala104ser125}$, IL-2$_{ser125}$, IL-2$_{ala104}$, IL-2-$_{ala104ser125}$, des-ala$_1$IL-2, or des-ala$_1$IL-2$_{ala104}$.

6. The composition of claim 1 wherein the immunotoxin comprises an antibody that selectively binds to human breast or ovarian cancer cells and is IgG or IgM.

7. The composition of claim 6 wherein the immunotoxin comprises an antibody that does not bind to blood cells, has a breast tumor binding range of at least 0.25, or has a breast cancer cell line binding range of greater than or equal to 0.25, and has a normal tissue reactivity equal to or less than 0.09.

8. The composition of claim 7 wherein the immunotoxin comprises an antibody that is selected from the group consisting of: 260F9, 280D11, 245E7, 520C9, 113F1, 226B2, 454C11, 2G3, 33F8, 317G5, 369F10, 9C6, 35E10, 106A10, 387H9, 421E8, 451C3, 454A12, 650E2, 741F8, 759E3, and antibodies that are functionally equivalent to a member of said group.

9. The composition of claim 8 wherein the immunotoxin comprises an antibody that is selected from the group consisting of 260F9 and 520C9, and antibodies that are functionally equivalent to a member of said group.

10. The composition of claim 1 wherein the immunotoxin comprises a recombinant rich A chain.

* * * * *

Exhibit 6

Fullerene (C_{60}) immunoconjugates: interaction of water-soluble C_{60} derivatives with the murine anti-gp240 melanoma antibody†

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The first fullerene (C_{60}) immunoconjugates have been prepared and characterized as an initial step toward the development of fullerene immunotherapy (FIT).

The field of biomedicine offers a promising arena for new applications of fullerene materials.[1] Water-soluble C_{60} derivatives are now commonplace,[2] and the discovery that water-soluble C_{60} derivatives can cross cell membranes[3] and even produce transfection[4] has accelerated interest in using C_{60} for diagnostic and therapeutic medicine. Although fullerene toxicity is of some concern, several water-soluble C_{60} derivatives have shown acceptable cytotoxicity for drug-delivery applications.[5]

A number of water-soluble C_{60} derivatives have been suggested for various medical applications. These applications include neuroprotective agents,[6] HIV-1 protease inhibitors,[7] bone-disorder drugs,[8] transfection vectors,[4] X-ray contrast agents,[9] photodynamic therapy (PDT) agents,[10] and a C_{60}–paclitaxel chemotherapeutic.[11] In addition, endohedral metallofullerenes have demonstrated potential as radiopharmaceuticals[12] and MRI contrast agents.[13] Fullerene-based micelles have also been developed as a drug delivery system.[14] To date, however, only the bone-drug application has involved biological targeting of a C_{60}-based material,[8] even though drug targeting is a desirable, if not essential, component of all drug-delivery strategies.

There is now a large body of literature regarding the development of cell-targeted delivery of agents for imaging and therapeutic applications.[15] Growth factors, cytokines and antibodies have all been extensively studied for their abilities to deliver payloads to the surface and the cytoplasm of target cells. The antibody designated ZME-018 targets the gp240 antigen (also known as the high molecular weight melanoma-associated antigen, HMWMAA) found on the surface of >80% of human melanoma cell lines and biopsy specimens.[16] This antibody has previously been extensively used in clinical imaging trials[17] and for the delivery of toxins, cytokines and other therapeutic agents to melanoma cells *in vitro* and *in vivo*.[18] Immunoconjugates containing ZME-018 are rapidly internalized into melanoma cells in culture.[19] Moreover, these conjugates effectively localize into melanoma xenografts after systemic administration and demonstrate impressive cytotoxic effects against established tumors in orthotopic models.[20]

In this communication, we report the synthesis and characterization of a new water-soluble C_{60} derivative (Fig. 1a) designed to covalently attach to proteins such as ZME-018 as an initial step toward targeted fullerene immunotherapy (FIT). A single-drug chemotherapeutic agent such as a recently reported C_{60}–paclitaxel conjugate[11] might be employed for FIT, but the real advantage of FIT over other targeted therapeutic agents is the potential for the attachment of multiple (and possibly different) drugs to the C_{60} scaffold in order to create targeted, single-dose "drug cocktails".

Several reports have been published regarding C_{60} interactions with large biomolecules.[21] C_{60} derivatives have been developed to bind myoglobin,[21a] form electrostatic interactions with cytochrome c,[21b,c] induce protein clusters and complexes in human serum albumin,[21d,e] and enhance catalytic activity *via* conjugation with the serine protease, subtilisin.[21f] Finally, one study has reported the X-ray crystal structure of a C_{60}-specific monoclonal antibody.[21g] Together, these studies suggested to us the possibility of creating a C_{60}–antibody conjugate as a proof-of-principle step towards FIT.

Fluorescence spectroscopy and transient absorption spectroscopy have previously been used to detect dendritic C_{60} interactions with cytochrome c.[21c] These spectroscopic probes have the advantage of monitoring C_{60} without interference from the biomolecule. In particular, triplet → triplet (T–T) absorption provides a method to sensitively and selectively monitor C_{60} derivatives through their known spectral and kinetic signatures.[22] We therefore use transient and ground state absorption measurements to track the fullerene components in synthesized immunoconjugates.

The two C_{60} derivatives shown in Fig. 1 were used in this study. A monoadduct of C_{60}–SPDP (without the water-solubilizing

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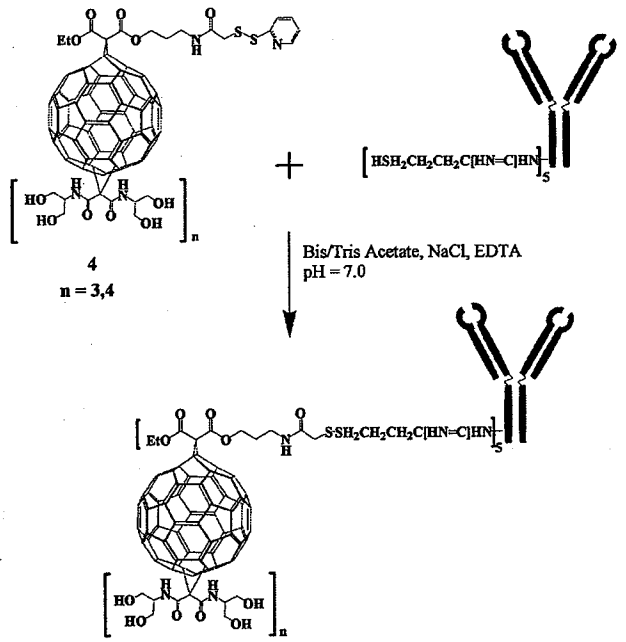
† Electronic supplementary information (ESI) available: Experimental and instrumentation detail. See DOI: 10.1039/b601717g

Fig. 1 The water-soluble fullerene (C_{60}) derivatives.



Scheme 1 Schematic representation showing the formation of the C_{60} immunoconjugate from C_{60}–SPDP (C_{60} and antibody figures not to scale).

malonodiserinolamide groups of Fig. 1a) was first prepared (see ESI†) to test the feasibility of attaching the cross-linker, *N*-succinimidyl-3-(2-pyridyldithio)propionate (or SPDP),[23] to C_{60}. Water-solubilizing malonodiserinolamide groups were then first attached to C_{60}, followed by the SPDP moiety in Fig. 1a to provide a cross-linking agent for the ZME-018 antibody. A water-soluble derivative of C_{60}–SPDP was found to be necessary to allow an interaction with the antibody.

Coupling of the C_{60}–SPDP to the antibody (for ratios of 1 : 1, 5 : 1 and 10 : 1) was then accomplished by reacting ZME-018 with 2-iminothiolane, which added an average of five thiol groups to the F_c fragment,[24] each of which can form a new disulfide bond with the SPDP sidearm of C_{60}–SPDP (Scheme 1). The coupling was performed in a salt solution to minimize fullerene aggregation.[21d] Products were purified by size-exclusion chromatography and then examined by transient absorption spectroscopy (ESI†). As shown in Fig. 2a, the C_{60} core's 690 nm T–T spectral signature was clearly present with intensities reflecting the reactant ratio. However, it was unclear whether covalent bonds had formed between C_{60}–SPDP and ZME-018. Therefore, the related water-soluble C_{60}–Ser derivative (Fig. 1b),[26] was substituted for C_{60}–SPDP in the reaction schemes with ZME-018 (10 : 1 C_{60}–Ser : ZME-018). To our surprise, results for the C_{60}–Ser derivative mirrored those of C_{60}–SPDP. This implies that C_{60}–(ZME-018) conjugate formation may not require covalent bond formation.

Our quantitative characterization began with BioRad protein assays, which showed that the concentration of ZME-018 in the chromatographically purified samples was 0.40 μM for C_{60}–SPDP–(ZME-018) and 0.36 μM for C_{60}–Ser–(ZME-018) (see ESI†). To find the corresponding fullerene concentrations in these conjugates, we used UV-vis spectroscopy. At 440 nm, the molar absorptivity of C_{60}–Ser far exceeds that of ZME-018. The conjugate's measured 440 nm absorbance (ESI†) directly showed a C_{60}–Ser concentration of 15 μM, implying that the ratio (C_{60}–Ser) : (ZME-018) was 42 : 1.[25] Spectral analysis of the C_{60}–SPDP–(ZME-18) conjugate was more complex because absorption bands of C_{60}–SPDP at 440 nm are not intense enough for determining concentrations <20 μM and at lower wavelengths (<350 nm) there is an overlap of absorption bands from the antibody. To account for this, we first prepared a reference solution containing only 0.40 μM ZME-018. As shown in Fig. 2b, this solution has significant absorption at 282 nm. We then added C_{60}–SPDP until the absorbance of the mixture near 282 nm matched that of the C_{60}–SPDP–(ZME-18) immunoconjugate known to contain a 0.40 μM concentration of antibody. The upper traces in Fig. 2b show spectra of this mixture and the conjugate. From the amount of C_{60}–SPDP used to prepare the matching mixture, we deduced a C_{60}–SPDP concentration of 6 μM in the conjugate, corresponding to a (C_{60}–SPDP) : (ZME-018) molar ratio of 15 : 1.

Enzyme-linked immunosorbent assay (ELISA) binding curves using antigen-positive cells as targets gave mid-points of 1.2 nM for the C_{60}–SPDP–(ZME-018) immunoconjugate, 26 nM for the C_{60}–Ser–(ZME-018) immunoconjugate, and 724 nM for a non-specific, isotype-matched murine IgG antibody used as a control (ESI†). Amazingly, the C_{60}–SPDP–(ZME-018) conjugate demonstrated binding midpoints similar to the non-conjugated ZME-018 antibody (mid-point of 0.46 nM), even though 8% (by weight) of the immunoconjugate is fullerene. However, the non-covalently bound C_{60}–Ser–(ZME-018) conjugate, consisting of 17% (by weight) fullerene, exhibited a much lower affinity than C_{60}–SPDP–(ZME-018). Regardless, the C_{60}–Ser–(ZME-018) conjugate was still a factor of 30 more effective in binding the target than was the control.

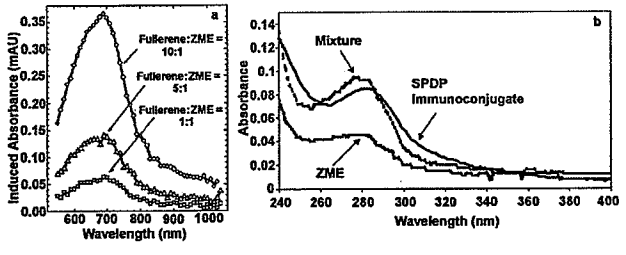


Fig. 2 (a) T–T spectrum of C_{60}–SPDP–(ZME-018) immunoconjugate prepared with three different ratios of fullerene to antibody, after chromatographic purification. (b) UV absorption spectra of 0.40 μM ZME-018, the C_{60}–SPDP–(ZME-018) immunoconjugate (chromatographically purified), and an unreacted mixture of the two components.

Fig. 3 TEM images of (a) ZME-018 antibody and (b) C_{60}–Ser–(ZME-018) immunoconjugate. The scale is the same for both frames; scale bar length is 20 nm. The solid curved feature in the image is the lacy carbon grid material.

To visualize the two C_{60} immunoconjugates, TEM images of both were obtained on a lacy carbon grid. Comparative images of the ZME-018 antibody and the immunoconjugate are shown in Fig. 3 (An image of C_{60}–SPDP–(ZME-018) and experimental details are presented in the ESI†). Fig. 3 shows that the free antibody appears to have a clear globular structure ~60 nm in diameter, whereas the image of the C_{60}–Ser immunoconjugate is also globular but 4–5 times larger in diameter. In addition, the C_{60}–Ser immunoconjugate image reveals numerous dark spots scattered throughout the structure that can be attributed to small aggregates of C_{60}–Ser, ~2–5 nm in diameter. The larger C_{60}–Ser–(ZME-018) size may reflect disruption of hydrogen bonding networks inside the antibody or some aggregation effect.

The above experiments confirm that covalent bond formation is not necessary to form immunoconjugates of water-soluble C_{60} derivatives with an antibody, and that antibody to antigen binding is not significantly reduced for high C_{60} : antibody molar ratios (15 : 1). Future studies will explore the cancer cell biology of these new C_{60} immunoconjugates, as well as immunoconjugates derived from other fullerene-based nanostructures that have the potential for targeted imaging and therapy in medicine.[11,13,26,27]

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Exhibit 7

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Invited articles

Human High Molecular Weight-melanoma Associated Antigen as a Target for Active Specific Immunotherapy

—A Phase I Clinical Trial with Murine Antiidiotypic Monoclonal Antibodies—

Soldano Ferrone and Toshiro Kageshita

Introduction

Active immunotherapy of malignant diseases aims at stimulating the patient's immune system to mount an immune response against tumor associated antigens (TAA) which may eventually destroy tumor cells. The immunotherapy is nonspecific when it utilizes agents such as Bacillus Calmette Guèrin, interferons, and interleukins which stimulate the patient's immune system in a nonspecific way. The immunotherapy is specific when it stimulates the patient's immune system specifically against TAA. In the latter approach, which is the most desirable from an immunological viewpoint, the stimulatory agents have been, in the past, tumor cells or their extracts (1). More recently, the hybridoma methodology has facilitated the use of antiidiotypic antibodies, since the availability of anti-TAA monoclonal antibodies has overcome the difficulties caused by the complexity and heterogeneity of antibody populations in conventional antisera to generate and analyze antiidiotypic antibodies. Furthermore, the hybridoma methodology has eliminated the practical difficulties in preparing large amounts of antiidiotypic antibodies, with finely defined specificity and with well standardized characteristics, from conventional antisera. For the last few years, we have implemented a program to evaluate the usefulness of murine antiidiotypic monoclonal antibodies to establish active specific immunotherapy in patients with melanoma. In the present paper, we will first give some background about antiidiotypic antibodies and the rationale which underlies their use to induce immunity against tumor associated antigens. Then, we will discuss the reasons why we have selected melanoma to evaluate the usefulness of antiidiotypic antibodies to induce immunity against TAA. Finally, we will summarize the results of a phase I clinical trial, with murine antiidiotypic monoclonal antibodies, which we have been per-

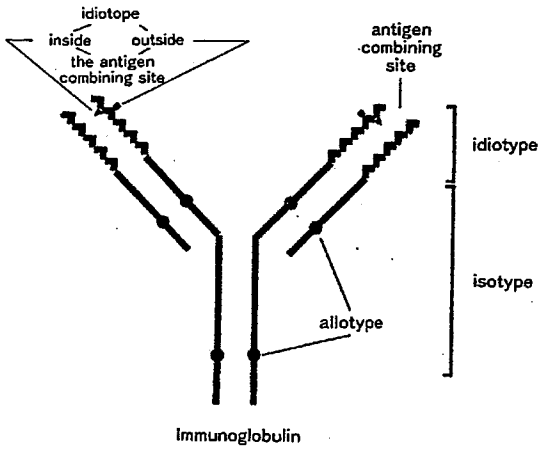

Fig. 1. Schematic representation of the immunoglobulin structure with the topographic distribution of isotypes, allotypes and idiotypes.

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Table 1. Characteristics of HMW-MAA

Characteristics		References
Cell distribution	membrane bound	(32)
Frequency in surgically removed melanoma lesions	at least 85% of the lesions tested	(21,26,27)
Distribution in normal tissues	restricted	(21,26,27)
Heterogeneity among lesions and among cells within a lesion	low	(17,25,27)
Density on tumor cells	between 1×10^5 and 2×10^6 antigenic sites/cultured melanoma cell	(12,19,24,28)
Susceptibility to modulation by antibodies	no	(16,19)
Susceptibility to modulation by lymphokines	low	(11,15,18,19,30)
Immunogenicity in patients with melanoma	low	(16,22)
Immunoscintigraphy in patients with melanoma	specific localization of radiolabeled monoclonal antibodies	(13,14,23,29)

forming in patients with advanced melanoma.

Active specific immunotherapy of malignant diseases with antiidiotypic antibodies to anti-TAA antibodies

Idiotypes represent the complex of determinants, *i.e.* idiotopes, expressed on the variable region of an antibody. Idiotopes may be immunogenic in syngeneic, allogeneic and xenogeneic combinations and may induce the formation of antibodies, which are referred to as antiidiotypic antibodies. Idiotopes may be located outside or within the antigen combining site of an antibody (Fig. 1). Among the latter, some may be located in the variable region in such a way that the corresponding antiidiotypic antibody reacts with the same portion of antibody which binds the corresponding antigen. These antiidiotypic antibodies may mimic the structure of the epitope of the original antigen and may be used to elicit immunity against it (2, 3). This property of antiidiotypes, in conjunction with their immunoregulatory properties, accounts for their use as immunotherapeutic agents to implement active specific immunotherapy in patients with malignancies.

Antiidiotypic monoclonal antibodies have both theoretical and practical advantages over purified TAA in approaches to elicit an immune response to TAA in patients with malignant diseases. Firstly, in several systems, antiidiotypic antibodies have been shown to stimulate silent clones unresponsive to nominal antigens (4, 5); therefore, antiidiotypic antibodies to anti-TAA antibodies may be able to elicit immunity even when TAA are not able to do it. Secondly, immunization with antiidiotypic antibodies to anti-TAA antibodies may overcome the resistance of tumor cells to immune T cell destruction caused by a lack of expression of HLA Class I antigens (6), since in murine systems cytotoxic T cells, induced with antiidiotypic antibodies to antiviral antigen antibodies, show less H-2 restriction at the level of the effector phase than T cells stimulated by viral antigens (7). Thirdly, since the function of most, if not all, TAA and their role in the transformation of cells are not known, antiidiotypic antibodies may eliminate the potential harmful side effects associated with the administration of TAA. Lastly, antiidiotypic monoclonal antibodies can be easily produced in purified form in large amounts utilizing hybridoma methodology, while purification with conventional biochemical and/or immunochemical methods of large amounts of TAA in an immunogenic form presents practical difficulties, at least until when procedures based upon molecular biology approaches become available.

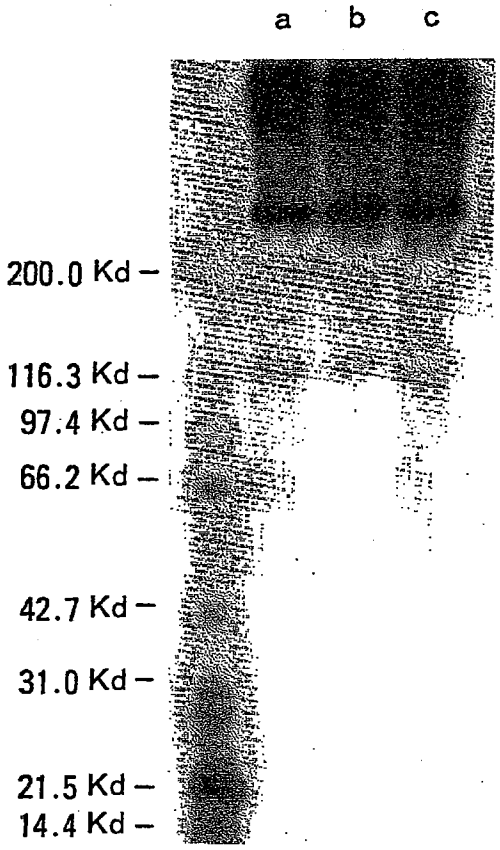
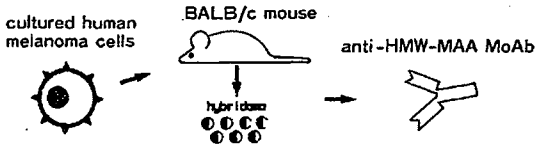
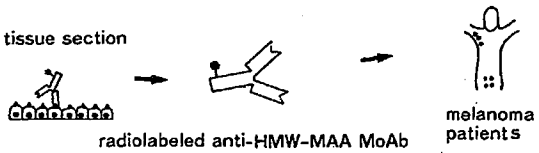


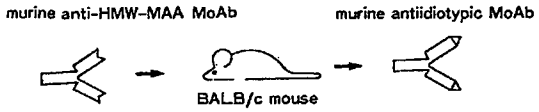
Fig. 2. SDS-PAGE analysis of components immunoprecipitated by the anti-HMW-MAA MoAb 225.28 (lane a), 736.74 (lane b) and TP 41.2 (lane c) from Colo 38 human melanoma cells. Colo 38 human melanoma cells ($1–2 \times 10^7$) were surface labeled with 1 mCi of ^{125}I utilizing the lactoperoxidase method (33). After 3 washings with PBS, cells were incubated with 3 ml of solubilizing buffer (50 mM Tris/150 mM NaCl/5 mM EDTA/0.5% Nonidet P-40 (Sigma Chemical Company, St. Louis, MO), pH 8.2, with 1 mM phenylmethylsulfonyl fluoride (Eastman Kodak Co., Rochester, NY) for 30 min at 4°C. Particulate material was removed by centrifugation at 100,000×g for 1 hr at 4°C. After preclearing with protein A-Sepharose 4B-CL suspension, cell extract (1×10^7 cpm) was mixed with 20 μl of Affi-gel 10 (Bio-Rad Laboratories, Richmond, CA) which had been coated with monoclonal antibody at the ratio of 2 mg/ml of gel according to the manufacturer's instructions. The mixture was incubated overnight with continuous rotation at 4°C. After washing, antigens were eluted from immunoadsorbent beads by boiling with 50 μl of electrophoresis sample buffer (34) and electrophoresed in a slab gel containing an acrylamide gradient of 3–15% and the Laemmli SDS buffer system (34). Gels were stained with Coomassie Brilliant Blue and processed for autoradiography utilizing a Kodak XAR-5 film. Myosin (200.0 Kd), β-galactosidase (116.3 Kd), phosphorylase b (97.4 Kd), bovine serum albumin (66.2 Kd), ovalbumin (42.7 Kd), carbonic anhydrase (31.0 Kd), soybean trypsin inhibitor (21.5 Kd) and lysozyme (14.4 Kd) (Bio-Rad, Rochville Centre, NY) were used as molecular weight standards.



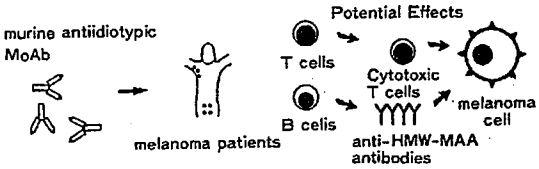
I. Development of murine monoclonal antibodies to human MAA



II. Selection of HMW-MAA as a target for immunotherapy



III. Development of antiidiotypic monoclonal antibodies to anti HMW-MAA monoclonal antibodies



IV. Immunotherapy with antiidiotypic monoclonal antibodies

Fig. 3. Schematic representation of the steps utilized to implement active specific immunotherapy with murine antiidiotypic monoclonal antibodies in patients with melanoma.

Table 2. Reactivity of anti-idiotypic MoAb MF11-30 in serological assays

Anti-idiotypic MoAb	Anti-HMW-MAA MoAb					
	225.28			TP41.2		
	$F(ab')_2$ binding assay[a]	Sandwich assay[b]	Inhibition of binding to melanoma cells	$F(ab')_2$ binding asay	Sandwich assay	Inhibition of binding to melanoma cells
MF11-30	13,000[b]	13,900	92.6[c]	600	128	11.0
TK6-74[c]	328	200	<5	15,000	9,053	91.0

[a]It was performed as described by Kusama et al. (9).
[b]It was performed as described by Tsujisaki et al. (31).
[c]The anti-idiotypic MoAb TK6-74 was elicited with the anti-HMW-MAA MoAb TP41.2.
[d]cpm.
[e]Percent inhibition.

Rationale for the selection of malignant melanoma for testing murine antiidiotypic monoclonal antibodies

To test the usefulness of murine antiidiotypic monoclonal antibodies to implement active specific immunotherapy in patients with solid tumors, we have selected human melanoma as a model system for the following three reasons. Melanoma is the most likely among human malignancies to benefit from immunotherapeutic approaches because of the potential role of immunological factors in the pathogenesis and clinical course of the disease (8). Furthermore, since very little, if any, progress has been made in the area of therapy of melanoma, it is justified from an ethical view point to implement trials based on novel approaches and reagents. Lastly, with murine monoclonal antibodies we have identified a melanoma associated antigen, referred to as high molecular weight-melanoma associated antigen (HMW-MAA) (Fig. 2), which meets the criteria to be an appropriate target to develop immunotherapeutic approaches to melanoma (Table 1). The steps we have followed to implement a phase I clinical trial in patients with stage IV melanoma are schematically shown in Fig. 3.

Table 3. Patients' characteristics

Number:	24
Sex:	13 females, 11 males
Age:	47 (34–74)
Karnofski performance status:	70% (60–90)

Development and characterization of the murine antiidiotypic MoAb MF11-30 to the syngeneic anti-HMW-MAA MoAb 225.28

The MoAb MF11-30 is secreted by a hybridoma constructed with splenocytes from a BALB/c mouse immunized with anti-HMW-MAA MoAb 225.28 according to a schedule similar to the one we have previously utilized to developed syngeneic antiidiotypic antisera to murine anti-HMW-MAA monoclonal antibodies (9). Briefly, purified MoAb 225.28 (200 μg), which had been coupled to keyhole limpet hemocyanin by glutaraldehyde fixation, was used as immunogen. The peritoneal route was

Table 4. Immunization schedule

Day	0	7	14	21	28	35	42	49	56	63	70
Immunizations	x	x			x	Further boosting is based on level of antiantiidiotypic antibodies					
Bleedings	x	x	x	x	x	x	x	x	x	x	x

• Double determinant immunoassay to detect mouse Ig in sera from patients injected with murine antiidiotypic MoAb MF11-30

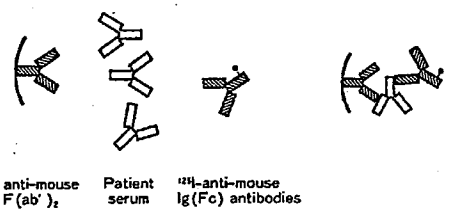


Fig. 4. Schematic representation of the double determinant immunoassay used to detect mouse Ig in sera from patients injected with murine antiidiotypic MoAb MF11-30.

Patient's serum was added to microtiter plates coated with goat anti-mouse IgG, $F(ab')_2$ antibodies. At the end of a 4 hr incubation at 4°C, plates were washed 3 times and added with ^{125}I-labeled anti-mouse Ig (Fc) antibodies. Incubation was continued for 2 hrs at 4°C. Plates were washed 5 times with PBS-Tween 20, wells were cut and bound radioactivity was measured in a γ counter.

• Binding assay to measure anti-mouse Ig antibodies in sera from patients injected with murine antiidiotypic MoAb MF11-30

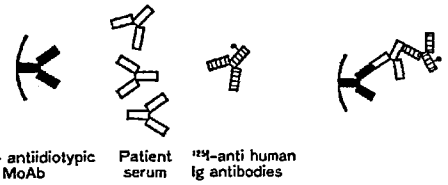


Fig. 5. Schematic representation of the binding assay to measure anti-mouse Ig antibodies in sera from patients injected with murine antiidiotypic MoAb MF11-30.

Patient's serum was added to microtiter plates coated with murine antiidiotypic MoAb and incubated for 4 hrs at 4°C. Plates were then washed 3 times with PBS-0.05% Tween 20 and added with ^{125}I-labeled anti human Ig antibodies. Incubation was continued for 2 hrs at 4°C. Plates were washed 5 times with PBS-Tween 20. Wells were cut and bound radioactivity was counted in a γ counter.

used for injections. Complete and incomplete Freund's adjuvant was used to prime and boost the mouse on days 0 and 7, respectively. On day 14, the mouse was boosted with MoAb 225.28 in phosphate-buffered saline, pH 7.2. Three days later, the mouse was sacrificed and the spleen was removed. Hybridization of splenocytes with murine myeloma cells P3-X63-Ag8.653 and subcloning were performed according to standard procedures (10). The fusion generated 293 hybridomas. Screening of supernatants with MoAb 225.28 in serological assays showed that 16 reacted in the $F(ab')_2$ binding assay, 4 in the sandwich assay and 4 inhibited the binding of MoAb 225.28 to HMW-MAA bearing melanoma cells. The MoAb MF11-30 showed strong reactivity in the three assays (Table 2). Testing of MoAb MF11-30 with a panel of monoclonal antibodies to distinct determinants of HLA Class I and Class II antigens has detected reactivity only with the MoAb 225.28. Therefore, the MoAb MF11-30 recognizes a private idiotope which is located within the antigen combining site of MoAb 225.28.

A phase I clinical trial with antiidiotypic MoAb MF11-30, elicited with anti-HMW-MAA MoAb 225.28 in patients with stage IV melanoma

The MoAb MF11-30, at doses ranging between 500 μg and 4 mg/injection, was injected intradermally into 24 patients with stage IV melanoma. Information about their clinical characteristics is summarized in Table 3. The immunization schedule has varied slightly among the patients: most of them have were immunized on days 0, 7 and 28 with additional injections given if antiantiidiotypic antibodies were not induced or when their titer declined (Table 4). The maximum amount of MoAb MF11-30 injected to a patient, thus far, has been 21 mg; the longest time of treatment has been 21 months. Serum has been harvested from each patient at weekly intervals and tested for its content of mouse immunoglobulins, of anti-mouse Ig antibodies and antiidiotypic antibodies to MoAb MF11-30, utilizing the assays schematically represented in Figs. 4, 5 and 6. Representative results of the kinetics of the development of anti-mouse Ig antibodies and of antiidiotypic antibodies are shown in

• Inhibition assay to measure antiantiidiotypic antibodies in sera from patients injected with murine antiidiotypic MoAb MF11-30

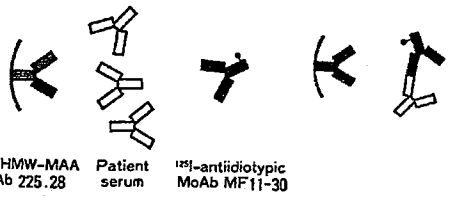


Fig. 6. Schematic representation of the inhibition assay to measure antiantiidiotypic antibodies in sera from patients injected with murine antiidiotypic MoAb MF11-30.

Patient's serum was incubated overnight at 4°C with ^{125}I-labeled antiidiotypic MoAb MF11-30. The mixture was then added to microtiter plates coated with anti-HMW-MAA MoAb 225.28. At the end of a 2 hr incubation at 4°C, plates were washed 5 times with PBS-0.05% Tween 20, wells were cut and bound radioactivity was counted in a γ counter.

Table 5. Clinical response to administration of antiidiotypic MoAb MF11-30

Response	Patient #	Duration (weeks)
Inevaluable	5	
Complete response	0	
Partial response	1	64
Minor response or stabilization of disease	8	84,48,41,34, 31,21,20,2
Progression of disease	10	

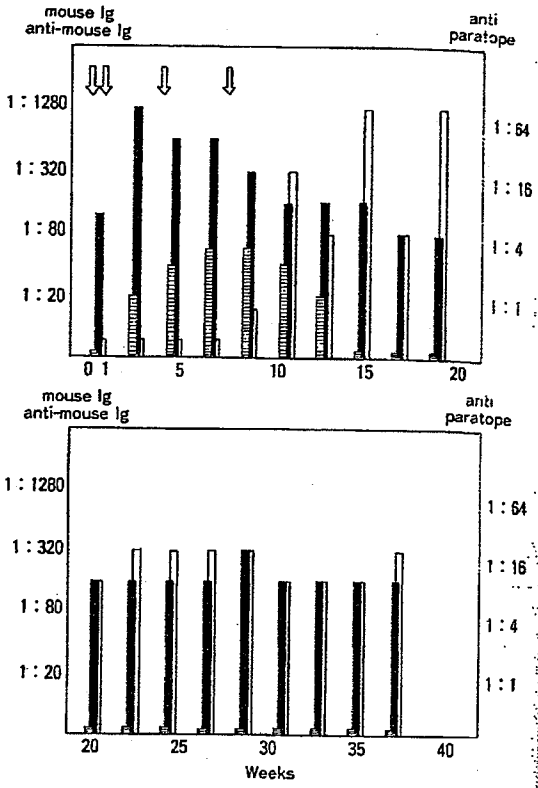


Fig. 7. Kinetics of the development of anti-mouse Ig antibodies and of antiantiidiotypic antibodies in the patient M.K. immunized with antiidiotypic MoAb MF11-30.

The antiidiotypic MoAb MF11-30 was purified from ascitic fluid by precipitation with caprylic acid and 2 mg were injected intradermally on the days indicated by arrows. Serum was harvested on the indicated days and tested for its content of mouse Ig (▤), anti-mouse Ig antibodies (■) and antiantiidiotypic antibodies (□).

Fig. 7. The following conclusions can be drawn:

1) Mouse Ig are detectable in patients' sera only in the early stages of the immunization. Once the titer of anti-mouse Ig antibodies is increased, it is likely that the injected mouse antibodies are complexed with anti-mouse Ig antibodies and become undetectable, either because of unavailability of determinants to react with the anti-mouse Ig antibodies in the double determinant immunoassay and/or because of rapid catabolism of the immunocomplexes.

2) Anti-mouse Ig antibodies with a titer up to 1:320 were detected in the sera from all the patients before immunization. The titer of the antibodies increased two weeks following the beginning of the immunization in the majority of patients.

3) Only 17 of the 24 injected patients developed anti-antiidiotypic antibodies to MoAb MF11-30, as indicated by the ability of their serum to inhibit by 90% the binding of MoAb MF11-30 to MoAb 225.28. The titer of

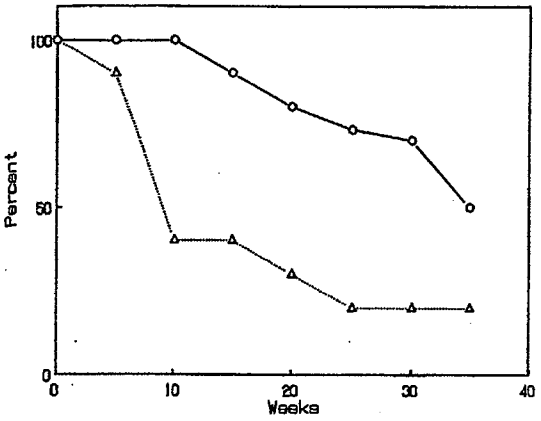


Fig. 8. Survival rate of patients who developed antiantiidiotypic antibodies with a titer of at least 1:8 (○) or lower than 1:8 (△) following immunization with the antiidiotypic MoAb MF11-30.

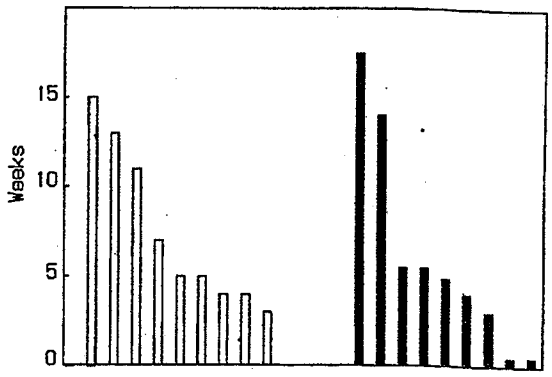


Fig. 9. Time interval between the first injection of antiidiotypic MoAb MF11-30 and the development of antiantiidiotypic antibodies in patients exhibiting partial response, minor response and stable disease (n=9) (□) and in those with progressive disease (n=10) (■).

these antibodies ranged between 1:2 and 1:256. Antiantiidiotypic antibodies appeared later than anti-mouse Ig antibodies.

From a clinical view point, it is noteworthy that no patient experienced any adverse reaction. In particular, injection of MoAb MF11-30 was not associated with any allergic and/or anaphylactic reaction, in spite of a high level of anti-mouse Ig antibodies. Although this is a phase I clinical trial, it is of interest that 6 patients showed a minor clinical response which lasted from a minimum of 2 to a maximum of 84 weeks (Table 5). A relationship was found between the titer of antiantiidiotypic antibodies to MoAb MF11-30 in patients' sera and their survival: the latter was longer when the titer of antiantiidiotypic antibodies was higher than 1:8 (Fig. 8). On the other hand, no relationship was found between patients' survival and interval between beginning of immunotherapy and appearance of antiantiidiotypic antibodies (Fig. 9).

Conclusions

This paper has described 1) the rationale underlying our decision to perform a clinical trial with murine antiidiotypic monoclonal antibodies to syngeneic anti-HMW-MAA monoclonal antibodies in patients with stage IV melanoma; 2) the development and characterization of the murine antiidiotypic MoAb MF11-30 which recognizes a private idiotope within the antigen combining site of the anti-HMW-MAA MoAb 225.28; and 3) the results of immunological and clinical investigations performed in 24 patients injected with the MoAb MF11-30.

The most reassuring aspect of our investigations has been the lack of side effects of repeated injections of murine antiidiotypic MoAb MF11-30 into patients with stage IV melanoma, in spite of the development of anti-mouse Ig antibodies. This finding, in conjunction with the regression of melanoma lesions, associated with the administration of antiidiotypic MoAb MF11-30, in 6 patients, justifies the continuation of clinical trials to optimize the immunization schedule with antiidiotypic monoclonal antibodies to anti-HMW-MAA monoclonal antibodies in terms of frequency of injections, dose of antiidiotypic monoclonal antibodies, injection of single antiidiotypic monoclonal antibodies or combinations of antiidiotypic monoclonal antibodies to distinct idiotopes expressed by one or different anti-HMW-MAA monoclonal antibodies, use of adjuvants and route of administration. The success of this study will depend on the extent of the characterization of the correlation between

changes in cellular and humoral immunity in patients with melanoma, following the administration of antiidiotypic monoclonal antibodies, and clinical response. The resulting information will contribute to our understanding of the mechanism(s) underlying the potential therapeutic usefulness of antiidiotypic monoclonal antibodies in patients with melanoma and may suggest criteria to select patients who are likely to benefit from this type of active specific immunotherapy.

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Exhibit 8

HUMAN GENE THERAPY 9:737–746 (March 20, 1998)
Mary Ann Liebert, Inc.

Retroviral Vector Targeting to Melanoma Cells by Single-Chain Antibody Incorporation in Envelope

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ABSTRACT

Two strategies for targeting recombinant retroviruses to melanoma cells were compared. One was to extend the tropism of an ecotropic envelope to human melanoma cells, the other was to enhance the tropism of an amphotropic envelope for melanoma cells. Chimeric retroviral envelopes, incorporating a single-chain antibody (ScFv) directed against high-molecular-weight melanoma-associated antigen (HMWMAA) at the amino terminus are correctly processed and incorporated into virions. ScFv-ecotropic envelope chimeras allow specific, but low-titer, targeting of HMWMAA-positive cells, when co-expressed with ecotropic envelopes. ScFv-amphotropic envelope chimeras bind specifically to HMWMAA-positive cells and allow preferential infection at high titer.

OVERVIEW SUMMARY

In most current cancer gene therapy, tumor cells are removed by biopsy and genetically modified *ex vivo* before being returned to the patient. This method is slow and costly and is limited to those patients from which a biopsy can be readily taken. For cancer gene therapy to have widespread clinical application it will be necessary to avoid *ex vivo* gene transfer. To do this, vectors must be developed that only transduce target tumour cells and not other surrounding cells. In this study our aim was to generate retroviral vectors that specifically infect melanoma cells.

INTRODUCTION

GENETIC MODIFICATION OF TUMOR CELLS to enhance their immunogenicity can be used to generate an antitumor immune response that will kill unmodified cells. This has been demonstrated in animal tumors, using cells modified to express molecules including cytokines (Fearon *et al.*, 1990; Gansbacher *et al.*, 1990; Dranoff *et al.*, 1993), foreign antigens (Sugiura *et al.*, 1988; Fearon *et al.*, 1988; Lukacs *et al.*, 1993), molecules that enhance antigen presentation (Hui *et al.*, 1984; Tanaka *et al.*, 1986), or the co-stimulatory antigen B7.1 (Chen *et al.*, 1994; Li *et al.*, 1994). In most subsequent clinical protocols, patient tumor biopsy cells have been genetically modified *ex vivo* then injected as an irradiated vaccine (Roth and Cristiano, 1997). Preparation of these patient-specific vaccines is slow and costly. Furthermore, the need to irradiate cells to prevent rapid growth of a tissue culture variant is likely to reduce the efficacy of many of the vaccines. An alternative is to deliver genes encoding immunostimulatory molecules or enzymes that activate prodrugs to tumor nodules *in situ*, by local injection of vector (Nabel *et al.*, 1993; Oldfield *et al.*, 1993; Blaese *et al.*, 1994; Rakhmilevich *et al.*, 1996). For this to be optimally effective, efficient gene delivery to tumor cells but not surrounding normal tissue is desirable. Here, we describe two approaches to target retroviral vectors to human melanoma cells by envelope modification.

The host range of a retrovirus is partly determined by an amino-terminal domain of the envelope protein, responsible for receptor binding (Heard and Danos, 1991; Battini *et al.*, 1995). Murine leukemia viruses (MLVs) with ecotropic tropism only infect rodent cells, because the binding domain of the ecotropic envelope recognizes an epitope present only in the rodent cationic amino acid transporter (CAT-1) (Closs *et al.*, 1993; Battini *et al.*, 1995). Amphotropic MLVs can infect cells of many species, because the envelope recognizes an epitope present in a phosphate transporter (Ram-1) that is widespread (Miller *et al.*, 1994). These two different tropisms allowed us to attempt two melanoma targeting strategies. One was to ex-

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tend the tropism of an ecotropic envelope to human melanoma cells; the other was to enhance the tropism of an amphotropic envelope for melanoma cells.

The melanoma surface antigen HMWMAA was chosen as a target because it is widely expressed on human melanoma cell lines and primary melanoma cells, but has a restricted distribution in normal tissues (Natali *et al.*, 1981; Wilson *et al.*, 1981). Several studies indicate that HMWMAA may play a role in metastasis and its expression is associated with a poor prognosis (Harper and Resifeld, 1983; Chattopadhyay *et al.*, 1991; Kageshita *et al.*, 1993). Antibodies directed against HMWMAA have been used clinically to target toxic agents to melanomas (Imai *et al.*, 1983; Yang and Resifeld, 1988; Spitler *et al.*, 1987; Oratz *et al.*, 1990). Recently, at least four mouse anti-id mAb which bear the mirror image of distinct antigenic determinants of HMWMAA have been used as immunogens in patients with advanced melanoma (Mittelman *et al.*, 1992, 1994; Ferrone *et al.*, 1993). We inserted the coding sequence for an ScFu which recognizes HMWMAA (Kupsch *et al.*, 1995) at the amino terminus of ecotropic and amphotropic MLV envelopes, leaving the endogenous receptor binding domain intact. We have previously shown that insertion of a Ram-1 binding domain at this position in the ecotropic envelope led to efficient viral binding to Ram-1 and infection of human cells while still allowing infection of rodent cells via CAT-1 (Cosset *et al.*, 1995a; Valsesia Wittmann *et al.*, 1996). We obtained selective infection of HMWMAA-positive cells with both of these chimeric envelopes; their relative merits for human gene therapy applications are discussed.

MATERIALS AND METHODS

Plasmids and chimeric envelopes

The DNA fragment encoding a ScFv, derived from the monoclonal antibody LMH2, which recognizes HMWMAA, was removed from the plasmid p211 (Kupch *et al.*, 1995) by digestion with *Sfi* I and *Not* I. ScLMO and ScLA were made by inserting this fragment into pEGFXMo1 (Ager *et al.*, 1996) and pEGF4070AL.1Fx (Nilson *et al.*, 1996), respectively, after removal of an epidermal growth factor (EGF) coding fragment by digestion with *Sfi* I and *Not* I. The ALF plasmid, which expresses 4070A envelope, was described previously (Cosset *et al.*, 1995b). pSV2NEO plasmid (CLONTECH) was used as a helper plasmid for G418 selection of transfected cells.

Cells

TELCeB6 cells are derived from the human rhabdomyosarcoma TE671 cell line (ATCC CRL-8805) and harbor the MFGnlslacZ vector genome and an MLV-Gag-Pol expression plasmid, CeB (Takeuchi *et al.*, 1994). TELCeB6/MOF1 and TELCeB6/AF7 cell lines are TELCeB6-derived cell lines expressing ecotropic (Mo) and amphotropic (A) envelopes, respectively (Cosset *et al.*, 1995b). S1 and P.C are primary cell cultures from melanoma biopsies derived as described in Patel *et al.* (1994). Other cell lines used are TE671, murine NIH-3T3 (ATCC CRL-6361), Mv-1-Lu (MINK) (ATCC CRL-6584), and human melanoma BOWES (ATCC CRL-9607). All cells were grown in Dulbecco's modified Eagle's medium (DMEM; GIBCO-BRL) supplemented with 10% fetal calf serum (FCS).

Virus harvests

Envelope expression plasmids were transfected using lipofectamine (GIBCO-BRL) into TELCeB6 cells. Transfected cells were selected with phleomycin (50 μg/ml) and pools of phleomycin-resistant clones were used for virus production. In parallel, clones were isolated and analyzed. For harvesting viruses, producer cells were grown at 37°C until they become confluent. Cells were further cultivated at 32°C for 4–7 days by feeding fresh DMEM supplemented with 10% FCS every 2 days. The medium was replaced with serum-free Optimem (GIBCO-BRL) and the supernatant was collected 10–14 hr later. Virus harvests were filtered through 0.45-μm filters and frozen at −70°C for later analysis or concentrated by ultrafiltration (Millipore ultrafree-centrifugal filter device BIOMAX-50K). For concentration of the supernatants, the filters were treated with ethanol 70% for sterilization, washed with phosphate-buffered saline (PBS), and centrifuged at 3,000 × *g* for 30 min 4°C to remove the PBS. Supernatants from producer cells were then added to the filters and centrifuged at 3,000 × *g* for variable times (30 min to 2 hr) to reach the concentration factor required.

Immunoblots

Virus pellets and cell lysates were analyzed by Western blot using goat antisera against Rausher leukemia virus (RLV) gp70 (SU) and p30 (CA) proteins as described previously (Cosset *et al.*, 1995a,b).

HMWMAA expression and envelope binding

Target cells were washed in PBS and detached by 10–20 min incubation at 37°C with 0.02% EDTA in PBS. Cells were washed with PFA (PBS supplemented with 2% FCS and 0.02% sodium azide). For detection of HMWMAA expression, about 5×10^5 cells were incubated with LMH2 antibody (Kupsch *et al.*, 1995) or another monoclonal anti-HMWMAA antibody, CP/Mel.2 (Immune Systems LTD) for 1 hr at 4°C, washed with PFA and then incubated with anti-mouse immunoglobulin G-fluorescein isothiocyanate (IgG-FITC)-conjugated antibodies (Sigma). Cells were then washed once with PFA and twice with PBS and incubated with 20 μg/ml of propidium iodide for 2 min. Fluorescence of live cells was analyzed with a fluorescence-activated cell sorter (FACS) (FACScan; Beckton Dickinson). Similar results were obtained for all cell lines with the two antibodies. For envelope binding about 5×10^5 cells were incubated with viral supernatants for 30 min at 4°C, unless otherwise stated. Cells were then washed three times with PFA and incubated for 45 min with goat anti-RLV gp70 antibody diluted at 1:1,000 in PFA. Cells were washed three times in PFA and incubated with anti-goat IgG-FITC conjugate (Sigma). Cells were then washed once with PFA and twice with PBS and analyzed by FACS.

Viral infection

Target cells were seeded in 24-well plates at a density of 5×10^4 cells/well. Then, 24 hr later, virus dilutions were added with or without 4 μg/ml Polybrene and cells were incubated as indicated. Viral supernatant was then removed and the cells were grown in regular medium for 24–48 hr. X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) staining was per-

formed as previously described (Tailor *et al.*, 1993; Takeuchi *et al.*, 1994). For competition experiments, target cells were preincubated with LMH2 for 30 min at 37°C and then incubated with the viral supernatants containing LMH2 for an additional 30 min at 37°C. Viral supernatant was then removed and the cells were incubated in regular medium for 24–48 hr, and X-Gal staining was performed.

RESULTS

Construction of chimeric envelopes

Two chimeras based on ecotropic (Moloney MLV-SU) or amphotropic (4070A MLV-SU) envelopes were generated. A ScFv antibody recognizing HMWMAA (Kupsch *et al.*, 1995) was inserted at the amino acid position +1 of either envelope. Previous data suggested that insertion of a Ram-1 binding domain at this site in an ecotropic envelope enhances targeted titers by increasing postbinding event (Valsesia Wittmann *et al.*, 1996). Insertion of ScFvs at this site in an amphotropic envelope allows the Ram-1 binding site to remain accessible (Ager *et al.*, 1996). The ecotropic chimera (ScLMo) and the amphotropic chimera (ScLA) are shown in Fig. 1.

Incorporation of ScLMo envelopes into virions

The plasmid expression ScLMo was transfected into TELCeB6 cells that express MLV gag and pol proteins and carry a provirus encoding β-galactosidase (β-Gal) (Cosset *et al.*, 1995b). Cells transfected with ScLMo were then selected. A bulk population of ScLMo-transfected cells produced virus with a titer of 5×10^3 infectious units per ml (IU/ml) on NIH-3T3 cells, compared to 10^6 IU/ml produced by a bulk population of cells transfected with the same vector encoding Mo envelope. A clone of cells, TELCeB6/ScLMO, was then selected which produced a titer of 10^5 IU/ml on NIH-3T3 cells. Lysates of TELCeB6, TELCeB6/MOF1 (a cell line expressing Mo envelopes, counterpart of TELCeB6/AF7) (Cosset *et al.*, 1995b), and TELCeB6/ScLMo cells were then analyzed for envelope expression by Western blot using a polyclonal anti-RLV-SU antibody (Fig. 2A, C tracks). The ScFv-Mo chimeric envelope was expressed at a reasonable level at the predicted size of 100 kD for processed SU.

To determine whether the ScFv-Mo chimera was efficiently incorporated into virions, supernatants from the transfected cells were pelleted and analyzed for the presence of viral SU-gp70 by western blot (Fig. 2A, V tracks). The TELCeB6/MOF1 viral pellet samples was diluted 1/10 before loading. Chimeric ScFv SU protein was detected in TELCeB6/ScLMo supernatant indicating that the protein was incorporated into virions.

Specific binding of ScLMo to HMWMAA-expressing cells

Viral supernatants were incubated with NIH-3T3 cells, which do not express HMWMAA (Fig. 2B, top panels), and with the human tumor cell line TE671, positive for HMWMAA expression (Fig. 2B, bottom panels). As expected, the ecotropic

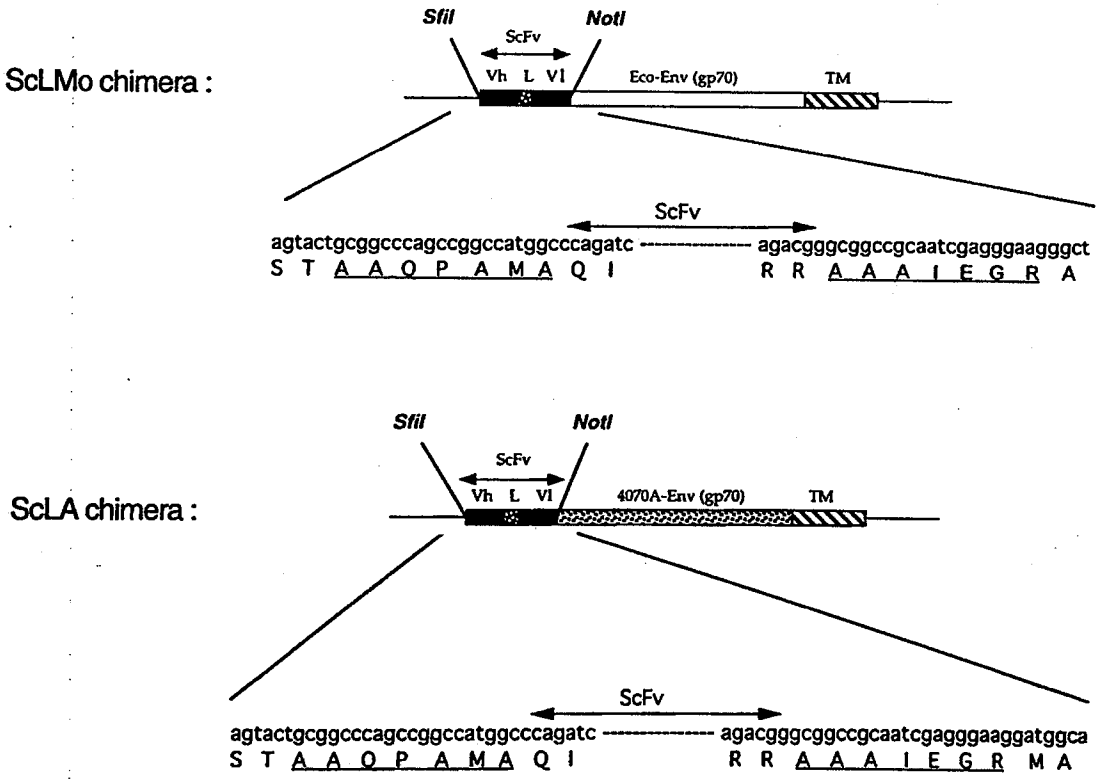

FIG. 1. Chimeric envelopes with a ScFv that recognizes high-molecular-weight melanoma associated antigen (HMWMAA) fused to ecotropic Moloney MLV-SU (ScLMo) or amphotropic 4070A MLV-SU (ScLA). Amino acid and nucleotide sequences at the junction between ScFv and MLV-SU are shown. Underlined sequences represent the amino acid linker synthesized to accommodate the cloning sites.

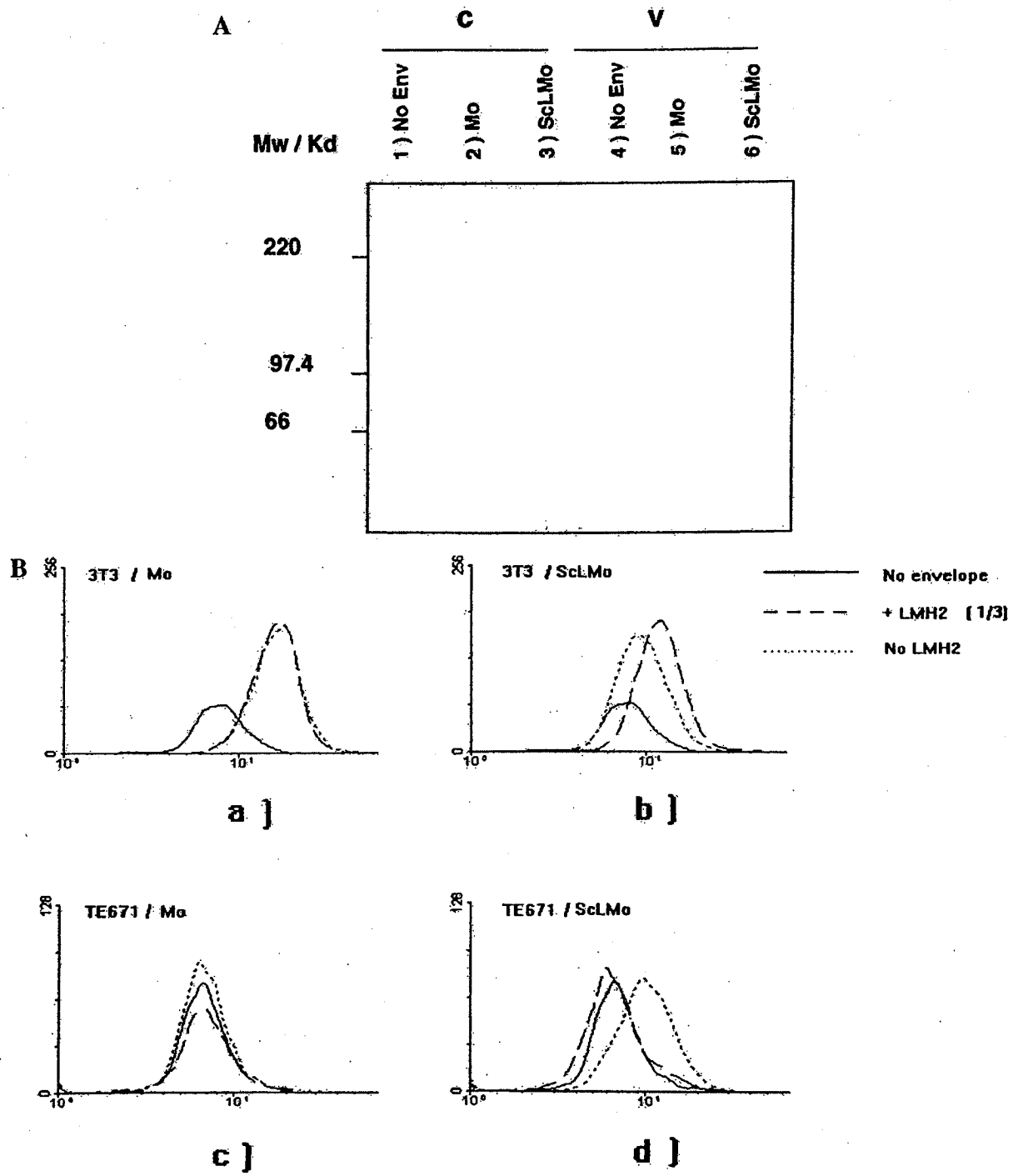

FIG. 2. Expression and binding of ScLMo chimeric envelope. A. Cell lysates (lanes 1–3) and supernatants (lanes 4–6) from parental TELCeB6 (lanes 1 and 4), TELCeB6/MOF1 (lanes 2 and 5), and TELCeB6/ScLMo (lanes 3 and 6) cells were separated by SDS-PAGE and transferred to a nitrocellulose membrane (Hybond ECL-Amersham). Immunoblots were incubated with anti RLV-SU antibody and developed with anti goat-HRP. B. Binding of viral supernatants from TELCeB6/MOF1 (a, c) and TELCeB6/ScLMo (b, d) cells to NIH-3T3 (a, b) and TE671 (c, d) cells. Bound virus was detected by the anti-RLV-SU antibody. Cells were incubated in viral supernatants for 60 min at 4°C. TELCeB6 supernatant was used to define background fluorescence (——). Binding of viral supernatants was measured in the presence (-----) or absence (·····) of LMH2. TE671 cells were positive for HMWMAA expression (peak channel shift = 52.7 when HMWMAA expression was analyzed by FACS analysis using CP/Mel.2 monoclonal antibody; see Materials and Methods); NIH-3T3 cells were negative (peak channel shift = 0).

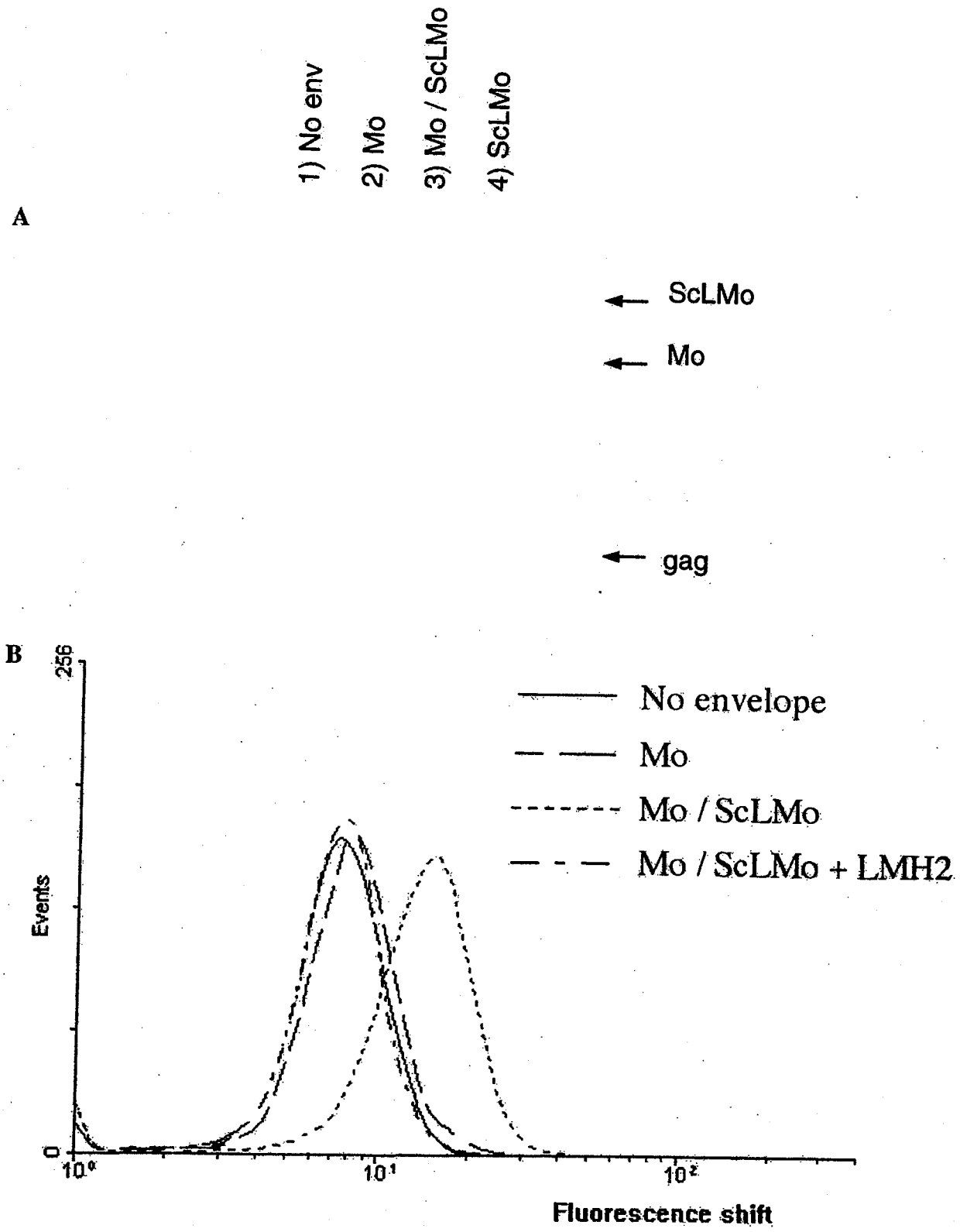

FIG. 3. Co-expression of Mo and ScLMo. A. Viral supernatants from TELCeB6 (lane 1), TELCeB6/MOF1 (lane 2), TELCeB6/MOF1/ScLMo which express both Mo and ScLMO envelopes (lane 3), and TELCeB6/ScLMo (lane 4) were separated by SDS-PAGE and then transferred to a nitrocellulose membrane (Hybond ECL-Amersham). The immunoblot was incubated with anti-RLV-SU (upper panel) and anti-RLV-CA (lower panel) antisera and developed with anti-goat-HRP. B. TE671 cells were incubated in TELCeB6/MOF1/ScLMo supernatant in the absence (——) or presence (——) of LMH2 antibody and in TELCeB6/MOF1 supernatant (– – –) for 60 min at 4°C. TELCeB6 supernatant was used to define background fluorescence (———).

TABLE 1. INFECTION BY VIRIONS EXPRESSING SCFV-MO ENVELOPES[a]

	Titer (lacZ IU/ml)					
Envelope:	ScLMo		Mo/ScLMo		Mo	
Target cell	+	−	+	−	+	−[b]
3T3	10^5	10^5	10^5	$1.5–10^5$	$1.2–10^5$	
TE671	0	0	90	420	0	0

[a]Viruses were incubated with target cells for 60 min at 4°C.
[b]Cells were preincubated (+) or not (−) with monoclonal antibody LMH2 against HMWMAA.

Mo envelopes could only bind NIH-3T3 cells (Fig. 2B, compare panels a and C). However, ScLMo envelopes bound both NIH-3T3 and TE671 cells; the binding of ScLMo to TE671 cells, but not NIH-3T3 cells, was inhibited by LMH2, the parental monoclonal antibody of the ScFv recognizing HMWMAA (Fig. 2B, compare panels b and d). This demonstrated that ScLMo was able to bind both HMWMAA and CAT-1. However, no infection of HMWMAA-expressing cells were observed with ScLMo enveloped viruses, even though the viral titer on NIH-3T3 cells after concentration was 10^6 IU/ml.

Production of viruses co-expressing ScLMo and Mo envelopes: infection of HMWMAA-expressing cells by extension of tropism

Previous studies suggested that co-expression of wild-type and chimeric envelopes allowed retargeted infection (Kasahara *et al.*, 1994; Somia *et al.*, 1995). We therefore co-transfected TELCeB6/MOF1 with the ScLMo expression plasmid and pSV2NEO. Stable transfected cells growing in G418 were then analyzed for Mo and ScLMo envelope production. Western blot

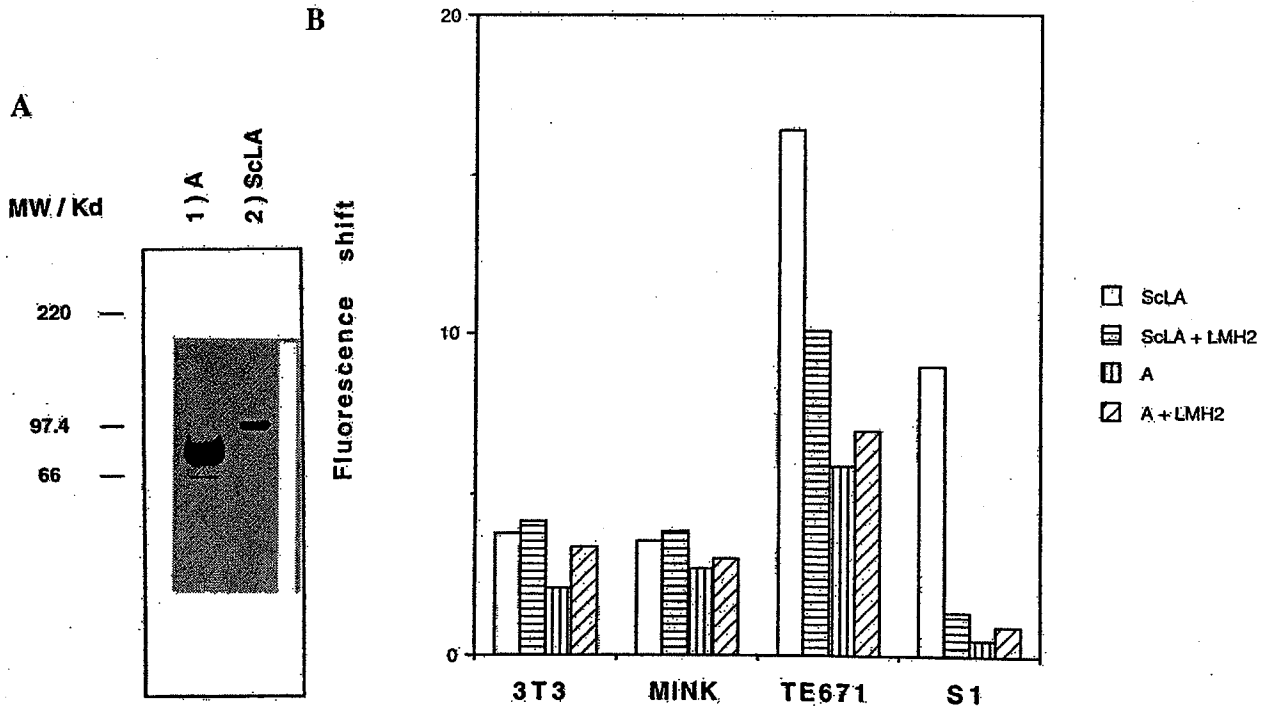

FIG. 4. Expression and target cell binding of ScLA chimeric envelope. A. Viral supernatants from TELCeB6/AF7 (lane 1) and TELCeB6/ScLA (lane 2) were separated by SDS-PAGE and transferred to a nitrocellulose membrane (Hybond ECL-Amersham). The immunoblot was incubated with anti RLV-SU antibody and developed with anti goat-HRP. B. Binding of concentrated supernatants from TELCeB6/AF7 (A) and TELCeB6/ScLA (ScLA) cells to HMWMAA-negative [NIH-3T3 (3T3) and Mv-1-Lu (MINK)] and positive cells (S1 and TE671) was analyzed in the presence or absence of LMH2. TELCeB6 supernatant was used to define background fluorescence and the relative peak channel shift compared to this value is plotted. Target cells were incubated at 37°C for 30 min with the 20-fold concentrated supernatants. Peak channel shift for HMWMAA expression (analyzed by FACS analysis using CP/Mel.2 monoclonal antibody) was: NIH-3T3 = 0; MINK = 0.4; TE671 = 52.7; S1 = 80.6.

TABLE 2. INFECTION BY 4070A AND ScLA VIRUSES

	S1/3T3	*TE671/3T3*	*P.C/3T3*	*BOWES/3T3*	*MINK/3T3*
A	0.046 (0.02)	1.05 (0.68)	0.003 (0.001)	0.00075 (0.00035)	0.29 (0.07)
ScLA	0.523 (0.2)	3.7 (1.47)	0.06 (0.02)	0.091 (0.074)	0.30 (0.14)

Values are average ratios of infection of HMWMAA positive and negative cells compared to 3T3 cells, calculated from two separate experiments. Standard error is shown in brackets. Peak channel shift for HMWMAA expression as analyzed by FACS using CP/Mel.2 antibody: 3T3 = 0; Mink = 0.4; TE671 = 52.7; S1 = 80.6; BOWES = 31.8; P.C = 60.5

analysis of pelleted supernatants showed a similar level of ScLMo incorporation into virions whether it was expressed alone or co-expressed with wild-type Mo envelopes (Fig. 3A). Supernatant from cells co-expressing Mo and ScLMo showed specific binding to HMWMAA-positive human tumor cells, which was inhibited by LMH2 (FIg. 3B). No binding was observed with supernatants from cells expressing Mo, ScLMo, or Mo/ScLMo when Mv-1-Lu (MINK) cells, negative for HMWMAA and CAT-1 expression, were used as target (data not shown).

Viruses co-expressing Mo and ScLMo envelopes were able to infect HMWMAA-positive TE671 cells, with the relatively low titer of 420 IU/ml after concentration (Table 1). These data were obtained with producer cells expressing an excess of Mo compared to ScLMo (Fig. 3A). However, a similar poor titer was also obtained from producer cells expressing ScLMo in excess (data not shown). The retargeted infection could be inhibited when TE671 cells were preincubated with LMH2. Viruses expressing Mo envelope alone, with an equivalent titer on NIH-3T3 cells could not infect TE671 cells. These data demonstrate that only Mo/ScLMo enveloped viruses are able to infect TE761 cells using HMWMAA as receptor. As the level of Mo and ScLMo envelopes expressed singly is similar to that in the co-expressing virions, these data demonstrate that the envelopes act cooperatively to permit infection.

Incorporation of ScLA into virions and binding to HMWMAA

We next attempted to achieve high-titer, targeted infection by incorporating the same anti-melanoma single chain antibody into the 4070A amphotropic envelope. Plasmids expressing ScLA and 4070A (AFL; Cosset *et al.*, 1995b) envelopes were transfected into TELCeB6 cells and stably transfected cells were selected. Supernatants from these bulk populations were pelleted and analyzed for the presence of viral SU-gp70 by western blot using anti-RLV-SU antibody (Fig. 4A). The level of ScLA chimera detected was approximately five-fold lower than that of the 4070A envelope. Supernatants from the 4070A- and ScLA-expressing cells were then incubated with NIH-3T3, Mv-1-Lu (MINK), TE671, and S1 (a primary cell line cultured from a melanoma biopsy) cells in the presence or absence of LMH2 (Fig. 4B). Binding of the 4070A envelope to the three cell lines was not affected by LMH2. ScLA chimeric envelopes could bind to NIH-3T3, TE671, and S1 cells, but in the case of HMWMAA-expressing cells (TE671 and S1) this binding was inhibited when LMH2 was present. Therefore, ScLA can bind to both Ram-1 and HMWMAA. Little binding of 4070A to S1 was detected and considerably greater binding of ScLA to S1 was observed in the absence of LMH2. These data suggest that such primary tumor cells express high levels of HMWMAA compared to Ram-1.

Preferential infection of HMWMAA-positive cells by ScLA-enveloped viruses

To determine if the ability of ScLA-enveloped viruses to bind to HMWMAA allows targeting of positive cells, we infected HMWMAA-positive cells (BOWES, TE671, S1, and P.C) and negative cells (NIH-3T3 and MINK) with ScLA and 4070A viruses. The titer of the two viruses on NIH-3T3 cells was 4070A = 5×10^6–10^7 IU/ml and ScLA = 3×10^5–10^6 IU/ml. Because NIH-3T3 is HMWMAA-negative, we use a X/NIH 3T3 ratio (where X is the titer on the cell X and NIH-3T3 is the titer on NIH-3T3) as an indicator of the differential sus-

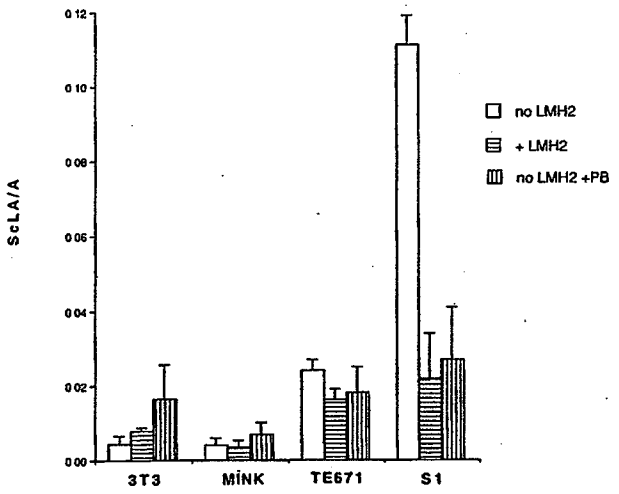

FIG. 5. Relative efficiency of infection by ScLA viruses. LacZ viruses bearing ScLA and A envelopes were used to infect NIH-3T3 (3T3), Mv-1-Lu (MINK), TE671, and S1 cells. The ratio of titers of ScLA and A enveloped viruses is shown for the experiments in the presence (+LMH2) and absence (no LMH2) of LMH2 and in the presence of 4 μg/ml of Polybrene (no LMH2 + PB). Target cells were incubated with LMH2 or medium at 37°C for 30 min, washed once with DMEM, and incubated with the viral supernatants at 37°C for 6 hr. Virus supernatant was replaced with fresh DMEM supplemented with 10% FCS and cells were incubated at 37°C for 48 hr prior to staining for β-Gal expression. Peak channel shift for HMWMAA expression (analyzed by FACS analysis using CP/Mel.2 monoclonal antibody) for the different cell lines used was: NIH 3T3 = 0; MINK = 0.4; TE671 = 52.7; S1 = 80.6.

ceptibility of HMWMAA-positive and -negative cells. The results are presented in Table 2; the actual titers of ScLA on the tumor cells were S1, 5×10^4 IU/ml; TE671, 5×10^5 IU/ml; P.C., 3×10^3 IU/ml; Bowes, 4×10^4 IU/ml. All the cell lines expressing HMWMAA showed higher X/NIH-3T3 ratios for ScLA than for 4070A viruses. This was greatest for the melanoma cell lines S1, P.C., and BOWES and reached 120 times higher for BOWES cells, indicating a preferential infection of HMWMAA positive melanoma cells by ScLA viruses. As expected, the X/NIH-3T3 ratio of HMWMAA negative cell line MINK was the same for 4070A and ScLA.

To demonstrate that the preferential infection of HMWMAA-expressing cells by ScLA viruses was due to HMWMAA binding, we determined the titer of the two viruses on two HMWMAA-positive cells (TE671 and S1) and two negatives (NIH-3T3 and MINK) in the presence or absence of LHM2. The ratio of titers ScLA/A was calculated in each case, as an indicator of the relative ability of the two viruses to infect each cell. Results are shown in Fig. 5. In the absence of LMH2, HMWMAA-expressing cells showed higher ScLA/A ratios than nonexpressing cells. When the same experiment was performed in the presence of LMH2, ScLA/A ratios were reduced, most dramatically in S1 cells. A similar reduction was observed when infections were performed in the presence of Polybrene, which suggests that the binding of ScLA viruses to HMWMAA enhancs infection of positive cells by increasing the rate of specific viral attachment to HMWMAA in a similar manner to the nonspecific enhancement of attachment by Polybrene. Infection of HMWMAA-positive cells also requires interaction between the envelope and Ram-1. This can be demonstrated using an antiserum against gp70 (SU) to block infection. For example, infection of TE671 cells by ScLA was reduced from 5×10^4 to $<10^2$ IU/ml in the presence of a goat antiserum against Rausher leukemia virus (RLV) gp70 (SU).

DISCUSSION

Although insertion of a Ram-1 binding domain at the extreme amino terminus of the ecotropic MLV envelope resulted in high-titer retargeting to human cells without ecotropic envelope co-expression (Cosset *et al.*, 1995a), insertion of an anti-HMWMAA ScFc into the same location allowed low-titer targeting to HMWMAA-positive cells, but only when ecotropic envelope was co-expressed. Both these chimeric envelopes are efficiently incorporated into virions and can redirect binding (Cosset *et al.*, 1995a, and this report). This demonstrates that insertion of an amino-terminal ligand that interacts with a natural viral receptor, such as Ram-1, permits efficient retargeting by enhancing a post-binding infection step. ScFv-HMWMAA interaction must fail to trigger a step that follows initial envelope-receptor interaction. Low-titer targeting to human cells was also obtained when an anti-MHC class I ScFv was inserted at the amino terminus of the ecotropic envelope (Marin *et al.*, 1996). Therefore, HMWMAA and MHC class I are both poorly permissive targets. Studies that inserted the EGF coding region at the amino terminus of the envelope showed that the EGF receptor fell into a third category of "inhibitory" receptor, which prevented viral infection (Cosset *et al.*, 1995a; Nilson *et al.*, 1996).

Other studies have constructed chimeric envelopes using either ligands (Kasahara *et al.*, 1994; Han *et al.*, 1995) or an ScFv recognizing the low-density lipoprotein (LDL) receptor (Somia *et al.*, 1995) in the ecotropic envelope. Infection of appropriate human cells was observed only when ecotropic envelope was co-expressed (Matano *et al.*, 1995). The somewhat higher titers reported for heregulin (Han *et al.*, 1995) or LDL receptor (Somia *et al.*, 1995) targeting, compared to that for HMWMAA reported here, might be due to a difference in envelope insertion site or a difference in the ability of these molecules to function as viral receptors. However, the highest titer of retargeting reported (10^4 IU/ml for LDL receptor) remains unsuitable for efficient *in vivo* gene delivery.

Our comparison of anti-HMWMAA ScFv insertion into ecotropic and amphotropic envelopes suggests that the latter approach may prove more realistic if high-titer targeting is to be achieved. These viruses preferentially infect HMWMAA-positive cells, particularly primary melanoma cells, which can probably be ascribed to higher expression of HMWMAA than Ram-1 on such cells. This suggests that they may be suitable for *in vivo* delivery to tumors, as many of the surrounding cells will be nondividing and therefore not susceptible to C-type retroviral infection. The level of specificity of surface targeting that we have achieved may well prove sufficient, particularly if it is combined with targeting at the level of vector expression (Vile *et al.*, 1994).

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Exhibit 9

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Antimelanoma Monoclonal Antibody–Ricin A Chain Immunoconjugate (XMMME-001-RTA) Plus Cyclophosphamide in the Treatment of Metastatic Malignant Melanoma: Results of a Phase II Trial

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Summary: Prior studies with the XMMME-001-RTA immunoconjugate composed of an antimelanoma monoclonal antibody and ricin A chain demonstrated some antitumor activity. However, almost all patients studied developed human antimurine antibodies and antiricin antibodies. In an effort to abrogate these host anti-immunotoxin immune responses and thus enhance antitumor activity, we treated 20 patients with the immunoconjugate plus a single dose of intravenous cyclophosphamide. An overall response rate of 20% was observed—predominantly in pulmonary and soft tissue nodules. There was no diminution in antibody responses against either the murine antibody or the ricin moiety. Further studies to elucidate the role of cyclophosphamide in monoclonal antibody therapy are planned. Key Words: Immunoconjugate—Antimelanoma monoclonal antibody—Ricin A chain—Human antimurine antibodies—Cyclophosphamide.

We have tested an antimelanoma monoclonal antibody conjugated to ricin A chain in patients with metastatic malignant melanoma. XMMME-001-RTA, an IgG_2 murine monoclonal antibody, recognizes two high molecular weight antigens of 220 kDa and greater than 500 kDa, and is conjugated to purified ricin A chain by SPDP reaction. Prior animal and phase I studies have demonstrated the safety of this agent (1,2). Phase II studies suggested potential clinical usefulness of this immunotoxin after a single course, with a small number of patients achieving durable partial remissions (3).

In these previous studies, all patients tested mounted a host antibody response against both murine immunoglobulin and ricin A chain moieties of the immunotoxin. Cyclophosphamide given with or shortly after sensitization to a new antigen has been shown in animal (4,5) and human studies (6) to blunt humoral responses to new antigens. In an animal model designed to test the effect of various immunosuppressive drugs on antibody responses, Santos et al. immunized rats with sheep red blood cells (SRBCs) and at various times in relation to immunization-administered cyclophosphamide, methotrexate, or 6-mercaptopurine (6). The animals were then bled periodically and peak anti-SRBC antibody titers were measured. Cyclophosphamide was a powerful inhibitor of the humoral response particularly when administered one or several days prior to immunization. In a comparison of the immunosuppressive capacity of drugs, cyclophospha-

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mide and methotrexate were far superior to 6-mercaptopurine. In an early clinical study (7), patients were immunized with either the V1 antigen (a purified polysaccaride) or a *Pasturella tularensis* vaccine. Immunosuppressive drugs including cyclophosphamide and 6-mercaptopurine were administered either prior to or following immunization. All patients who received cyclophosphamide (7 mg/kg i.v. daily for 7 days prior to antigen challenge) showed no rise in antibody titer while under observation.

More recent animal studies (8) used a Balb/c mouse model in which animals were sensitized with alloantigens comprised of spleen, thymus, and lymph node cells from C3H mice. Two dosages (20% LD_{50} and 60% LD_{50}) of a number of different immunosuppressive agents were administered at various times in relation to antigen challenge. Cyclophosphamide in doses of 102 and 306 mg/kg both showed strong suppression of antibody responses when given with or shortly after the immunizing antigen.

In an effort to enhance antitumor responses and abrogate the host anti-immunotoxin antibody response, cyclophosphamide was added to this antimelanoma immunotoxic protocol. A single large dose of cyclophosphamide (1,000 mg/m^2 i.v.) given immediately following immunotoxin infusion was selected based on preclinical and animal studies, so that the immunosuppressive agent was given essentially along with the potential antigen.

MATERIALS AND METHODS

Patients

Patients over age 18 years with histologically documented malignant melanoma with measurable metastatic disease were eligible for study. Other eligibility criteria included Karnofsky performance status ≥80% and life expectancy of at least 12 weeks. Patients were required to have adequate bone marrow, renal, and liver function. Prior systemic therapy for metastatic melanoma was allowed. Patients with resected, irradiated brain metastases and stable head computed tomography (CT) scan were eligible. This study was approved by the New York University Medical Center IRB and all patients signed written informed consent prior to treatment. Patients were ineligible if they had previously been treated with murine monoclonal antibodies or ricin A chain containing toxins.

Immunotoxin and Cyclophosphamide

The immunotoxin XMMME-001-RTA was provided by XOMA Corporation (Berkeley, CA, U.S.A.) (9). The immunotoxin is a murine monoclonal antibody of the IgG_{2a} subclass (MW 150,000±) to which ricin toxin A chain (RTA) (MW 30,000) is covalently coupled. The conjugation technique has been previously described (1,9). The ricin A chain is purified by affinity chromatography using an anti-ricin B chain column. Briefly, the antibody is activated with *N*-succinimidyl-3-(2-pyridyldithio)proprionate followed by addition of affinity-purified ricin A chain that has been reduced with dithiothreitol. The immunotoxin is then purified by gel chromatography. It is provided in a sterile, pyrogen-free formulation at a concentration of 1.0 mg/ml in 0.9% phosphate-buffered saline solution, pH 7.0. The binding specificity of the antibody as determined by enzyme-linked immunosorbent assay, radioimmunoassay, flow cytometry, and immunoperoxidase techniques demonstrates binding with all melanomas tested by frozen section and the majority of melanoma cell lines. There is no binding with other tumors or normal tissues with the exception of pigmented nevi and some cytoplasmic binding of vascular endothelium. Specificity of the ricin-conjugated immunotoxin is identical to that of the unlabeled antibody and binding activity is only minimally reduced by conjugation. Lots of 30 mg were shipped in 10 mg vials. Each patient was treated with a single lot of immunotoxin.

Commercially prepared cyclophosphamide was used.

Treatment Plan

Prior to each treatment, each patient had both a skin test and, if negative, an intravenous (i.v.) test dose. Skin tests were performed by subcutaneously injecting 0.1 ml of saline containing 0.01 mg of immunotoxin. Skin tests were considered to be negative if erythema and induration at the site were less than 5 mm in greatest diameter at 30 min. After a negative skin test, patients received an intravenous challenge with 1 ml containing 0.2 mg of immunotoxin. Patients were monitored closely and vital signs were recorded for 30 min following the i.v. test dose. If no adverse reaction developed, patients were then treated with an intravenous infusion of immunotoxin at a dose of 0.4 mg/kg in 150 cc of normal saline over 30–60 min. Thirty minutes

after the completion of immunotoxin infusion, patients received intravenous cyclophosphamide at a dose of 1,000 mg/m^2.

Physical examination, with measurement of indicator lesions, and laboratory tests were performed at baseline and weekly for 1 month on an outpatient basis and less frequently thereafter. Patients were followed for tumor response for a minimum of 8 weeks after the initiation of treatment. Laboratory tests included blood samples analyzed for complete blood count with white blood cell differential, platelet count, and serum chemistry tests including electrolytes, urea nitrogen, creatinine, and liver enzymes as well as albumin and total serum protein. Serum samples for quantitative determinations of human antimurine and antiricin immunoglobulins were obtained before treatment and at weekly intervals thereafter.

Assay for Human Antimurine and Antiricin Antibodies

The antibody response to immunotoxin components was measured in all patients by a previously described enzyme immunoassay method (1). Patients' sera were obtained prior to treatment and at weekly intervals thereafter. Appropriate serial dilutions (1:10 + 1:10^5) were prepared and added to microtiter plates that contained either the adsorbed murine antimelanoma monoclonal antibody or adsorbed ricin A chain. The plates were washed and incubated for 1 h at room temperature with goat anti-human IgG antibody or anti-human IgM conjugated to alkaline phosphatase (Zymed Laboratories, South San Francisco, CA, U.S.A.). Another wash was followed by addition of *p*-nitrophenyl phosphate (Sigma Laboratories, St. Louis, MO, U.S.A.). This reaction produced a color titration measured spectrophotometrically at 405 nm. Titration curves were generated for each serum sample and immune responses were expressed as a response ratio: the ratio of the end-point dilution of the serum sample showing maximum response to the end-point dilution of the pretreatment serum sample.

Evaluation of Tumor Responses

Patients were examined weekly for 4 weeks and then every 2 weeks for 4 weeks following treatment in order to evaluate tumor response. Palpable disease was assessed by weekly examination and direct measurement of the perpendicular diameters of all measurable nodules. CT scans and chest radiographs were used to evaluate visceral disease and were obtained every 4 weeks. Complete response was defined as the disappearance of all measurable tumor. Partial response was defined as a reduction of all measurable tumors by at least 50% of the sum of the product of the two greatest diameters present, in the absence of any new lesions or any tumor enlargement. Mixed response was a reduction in size of some measurable tumors by at least 50%, but either no change or progressive disease in other tumors. Minimal response was a reduction in size of less than 50% in some tumors. Stable disease was no objective change in all measurable tumors. Progressive disease was an increase in size of measurable tumors by at least 25% or the appearance of new lesions. The duration of response was defined from the date of therapy until the date of progressive disease, most recent follow-up, or death. All responses were required to persist for at least 30 days.

RESULTS

Patients

Twenty patients were entered. Their characteristics are detailed in Table 1. The median age was 58.5 years (range of 38–73 years). Twelve were male and 8 were female. Nine patients (45%) had received no prior treatment for metastatic melanoma, whereas 11 patients (55%) had been previously

TABLE 1. *Patient characteristics*

	No. (%)
Age	
Median (years)	58.5
Range	38–73
Sex	
Male	12 (60)
Female	8 (40)
No prior treatment	9 (45)
Prior treatment	11 (55)
Chemotherapy	7 (35)
Radiotherapy	5 (25)
Immunotherapy	6 (30)
Sites of metastatic disease	
Soft tissue/subcutaneous/lymph nodes	9 (45)
Lung	7 (35)
Liver	4 (20)
Spleen	5 (25)
Brain	2 (10)
Adrenal	1 (5)

No. of patients = 20.

treated with chemotherapy, radiation therapy, immunotherapy, or a combination of these. Most patients had more than one site of metastatic disease. The predominant areas of involvement included skin and soft tissue, lung, and liver. Two patients with resected and irradiated brain metastases were treated on this protocol.

Toxicity

All patients were evaluable for toxicity. Overall, the combination treatment was well tolerated and toxicity was manageable. Patterns of toxicity are outlined in Table 2. No patient had a positive reaction to the skin test dose. No patient developed hypotension, tachycardia, rash, hives, or wheezing during the intravenous test dose of immunotoxin. One patient had an episode of sneezing during the i.v. test dose with no other symptoms. After receiving approximately 30 cc of the intravenous infusion dose, he developed facial flushing, increased lacrimation, and swelling of the lower lip. He had no dyspnea, wheezing, stridor, rash, hypotension, tachycardia, or fever. The infusion was discontinued and the patient was given 50 mg of diphenhydramine by i.v. bolus. His symptoms resolved, and the immunotoxin infusion was resumed. The treatment was completed at a slower infusion rate and was well tolerated.

Other immediate toxicities from this regimen included nausea and vomiting in 18/20 (90%) patients in the first 24–48 h (mild in 13 patients, moderate in 5 patients), which was felt to be due to cyclophosphamide. Low-grade fevers were seen in 4/20 (20%) patients during the first 72 h after treatment. Most patients complained of constitutional symptoms consisting of fatigue, malaise, myalgias, and arthralgias during the first several days after treatment. This was reflected in a general decline in performance status by at least 10–20%, and resolved with return to baseline performance status by the middle of the second week after treatment.

Within the first 2 weeks following treatment, hypoalbuminemia was noted in 15/20 (75%) patients, manifested by decreases in serum albumin by less than 0.2 g/dl in 5 patients, ≥0.2–0.5 g/dl in 3 patients, and greater than 0.5 g/dl in 7 patients. Only five of these patients developed clinically evident peripheral edema: three patients had mild ankle swelling, one patient had lower extremity edema that was treated with oral furosemide, and one patient developed significant swelling of the left arm that had been the site of his primary melanoma and left axillary lymphadenectomy. The upper extremity edema was managed with oral diuretics, arm elevation, and an elastic arm stocking and resolved within 1 week. Three patients reported mild to moderate dyspnea—but pulmonary edema was not documented on chest radiographs and on no occasion were rales or wheezing appreciated on auscultation.

One patient became acutely ill within 24 h of treatment. She had a history of resected and irradiated brain metastases. A CT scan of the brain done less than 4 weeks prior to treatment showed no evidence of involvement, and her pretreatment neurologic exam was unremarkable. Nonetheless, she developed grand mal seizures on the evening following treatment. A repeat CT scan the next day revealed the presence of multiple new brain metastases. Furthermore, this patient remained hospitalized and developed grade 4 neutropenia, grade 3 anemia, fever, and sepsis. She had been heavily

TABLE 2. *Toxicity results*

Grade	None (0)	Mild (1)	Moderate (2)	Severe (3)	Life-threatening (4)
Fever	16	3	1	0	0
Fatigue	4	5	7	4	0
Malaise	11	9	0	0	0
Myalgias	10	8	2	0	0
Arthralgias	18	1	0	1	0
Dyspnea	17	2	1	0	0
Edema	15	3	1	1	0
Decreased albumin	5	5	3	7	0
Nausea/vomiting	2	13	5	0	0
Neutropenia	7	6	5	1	1
Anemia	17	1	1	1	0
Decrease in performance status	2	6	8	3	1
Seizures[a]	19	0	1	0	0

[a] See the discussion in the Toxicity section.

pretreated with multiple cytotoxic and myelosuppressive regimens. Despite a measureable partial response in her non-central nervous system tumor, the patient expired 43 days following treatment, related to nadir sepsis and brain metastases. There were no other deaths during the study period.

Clinical Responses

Clinical outcomes of responding patients are detailed in Table 3. There were no complete responses to treatment. Four (20%) partial responses were seen. One patient achieved a partial response as measured by disappearance of several pulmonary nodules and reduction of all others by at least 50%. Early response was noted on the chest radiograph within 1 month after treatment, and improvement continued over 2–3 months. This patient received a second immunotoxin treatment 4 months after his initial treatment and the lesions then stabilized for a total response duration of 1 year. During this time, the patient was clinically well, and worked regularly. A second patient had a partial response of skin, soft tissue, and lymph node disease including multiple tumors involving the gastrointestinal tract. He reported tenderness of the responding subcutaneous and soft tissue nodules during the first week after treatment. These became erythematous and warm, and over the next 2–3 weeks gradually became softer and smaller. Some nodules disappeared completely. The duration of response was 10 weeks. Two other patients had partial responses in subcutaneous and soft tissue nodules of 6 and 15 weeks duration. Of interest, responding nodules in general did not grow at the time of disease progression. Progressive disease was usually characterized by growth of nonresponding nodules or appearance of new metastases. One patient (5%) had a minor response characterized by a 30% reduction in a soft tissue pelvic mass of greater than 56 weeks duration. She has required no further treatment of her melanoma. Two patients (10%) had mixed responses in which there was a 50% decrease in the size of soft tissue metastases but progressive disease in visceral lesions. Three patients (15%) had stable disease throughout the study period and 10 patients (50%) had progressive disease.

Immune Responses

In 13 patients, pre- and posttreatment titers of human antimurine antibodies and antiricin antibodies were determined separately, and in seven patients antibody titers against the complete immunotoxin were measured. In all instances but one, antibody titers against the mouse immunoglobulin, ricin A chain, and whole immunotoxin rose after treatment. Patient #122 did not mount an antibody response against the ricin moiety of the immunoconjugate but did produce a response ratio of 7.5 in the human antimurine antibody (HAMA) response. Baseline, maximum end-point titers, and day 28 titers are shown in Tables 4–6. The maximum response ratios are displayed graphically in Fig. 1. The median HAMA response ratio was 1.25 (range of 3–100). The median response ratio to the ricin A chain component was 32 (range of 1–250). In the seven patients so tested, the median response ratio to the whole immunotoxin was 13.3 (range of 4.5–62.5).

The single dose of 1,000 mg/m^2 of cyclophosphamide used in this study neither abrogated the pro-

TABLE 3. *Clinical features of responding patients*

Patient no.	Age (years)/sex	Sites of disease	Prior therapy	Response	Duration (weeks)
121	73/M	Lung	None	PR	53
124	45/M	Soft tissue, skin, lymph nodes, GI tract	Chemotherapy	PR	10
130	59/F	Soft tissue, skin, lymph nodes, brain	Chemotherapy/RT	PR	6
138	73/F	Soft tissue, lymph nodes	None	PR	15
125	58/F	Lymph nodes	None	Minor	56+
133	46/M	Lymph nodes	None	Mixed	6 (progression in spleen, continued response in lymph nodes)
135	52/M	Soft tissue, skin, GI tract	None	Mixed	15 (progression in GI tract but continued response in skin and soft tissue)

PR, partial response.

TABLE 4. *Human antimurine antibody (HAMA) responses*

Patient no.	Baseline titer	Maximum end-point titer	Day 28 titer	Response ratio max./basline	Response ratio day 28/baseline
120	100	10,000	10,000	100	100
121	100	3,300	3,300	33	33
122	1,600	12,000	12,000	7.5	7.5
123	1,666	30,000	30,000	18	18
124	800	10,000	10,000	12.5	12.5
125	1,000	20,000	20,000	20	20
126	4,000	16,000	14,000	4	3.5
127	3,500	35,000	35,000	10	10
128	3,182	14,000	14,000	4.4	4.4
129	2,000	150,000	100,000	75	50
130	1,600	60,000	50,000	37.5	31.3
131	4,000	30,000	30,000	7.5	7.5
132	800	24,000	24,000	3.0	3.0

duction of these antibodies nor decreased the titers of the responses. The median and overall response ratios reported here are not significantly different than those previously published for treatment with the immunotoxin alone (1). Despite the appearance of the antibody response, one patient underwent repeat skin test and i.v. challenge that were both negative, and he was successfully retreated with immunotoxin without an allergic reaction.

DISCUSSION

This study was conducted to determine whether a single large dose of cyclophosphamide given immediately after immunotoxin administration would abrogate the immune response to the immunotoxin components. Suppression of the immune response was not achieved. An unexpected clinical observation, however, was that the tumor response rate in patients receiving this combination was as good as or better than that previously observed, despite the fact that the dose of immunotoxin administered was one-fifth of that used in previous studies. This suggests the possibility of a synergistic action between the immunotoxin and cyclophosphamide.

In a previous phase I trial, 1 complete response was observed in 21 evaluable patients (1). In a subsequent phase II study, there were 3 partial responses in 43 patients (3). Thus, in these studies, there were 4 responses in 64 patients as compared with 4 responses in 20 patients in the current study. This difference is of borderline statistical significance ($p = 0.07$); but it suggests that the addition of cyclophosphamide might improve the efficacy of the immunotoxin. It is unlikely that cyclophosphamide in the dose and schedule used in this trial resulted in the antitumor activity seen. However, it is possible that the cyclophosphamide modulated host lymphocyte subsets such that, rather than blunting humoral responses against the immunotoxin, it enhanced immune responses against melanoma. In a recent study by Uekun et al., the cyclophospha-

TABLE 5. *Human antiricin antibody (HARA) responses*

Patient	Baseline titer	Maximum end-point titer	Day 28 titer	Response ratio max./basline	Response ratio day 28/baseline
120	100	3,200	3,200	32	32
121	100	2,000	2,000	20	20
122	100	100	100	1	1
123	200	8,000	8,000	40	40
124	100	10,000	10,000	100	100
125	200	50,000	50,000	250	250
126	200	6,400	64,000	32	32
127	615	800	800	1.3	1.3
128	200	5,000	5,000	25	25
129	800	10,000	10,000	12.5	12.5
130	400	40,000	40,000	100	100
131	600	25,600	25,600	42.7	42.7
132	1,000	24,000	24,000	24	24

TABLE 6. *Human anti-immunotoxin antibody responses*

Patient no.	Baseline titer	Maximum end-point titer	Day 28 titer	Response ratio max./baseline	Response ratio day 28/baseline
133	400	12,800	12,800	32	32
134	2,000	12,800	9,000	6.4	4.5
135	800	24,000	24,000	30	30
136	5,000	60,000	60,000	12	12
137	3,000	24,000	24,000	8	8
138	3,000	40,000	40,000	13.3	13.3
139	800	50,000	50,000	62.5	62.5

mide congener, mafosfamid, markedly enhanced the target cell cytotoxicity of a ricin-conjugated monoclonal antibody in an in vitro clonogenic assay (10). The authors found that mafosfamid appeared to shorten the lag period seen in the immunotoxin inactivation of protein synthesis. They speculated that the mafosfamid effect might be related to (a) alteration of the chemical processing of the immunotoxin by target cells, (b) action at the ribosomal level, (c) increased sensitivity of the neoplastic cells because of a decline in aldehyde dehydrogenase activity, or (d) immunotoxin-induced acceleration of mafosfamid to reactive mustard and acrolein metabolites. Our study was not designed to examine such effects and therefore we did not collect data directed at addressing these questions. Further stud-

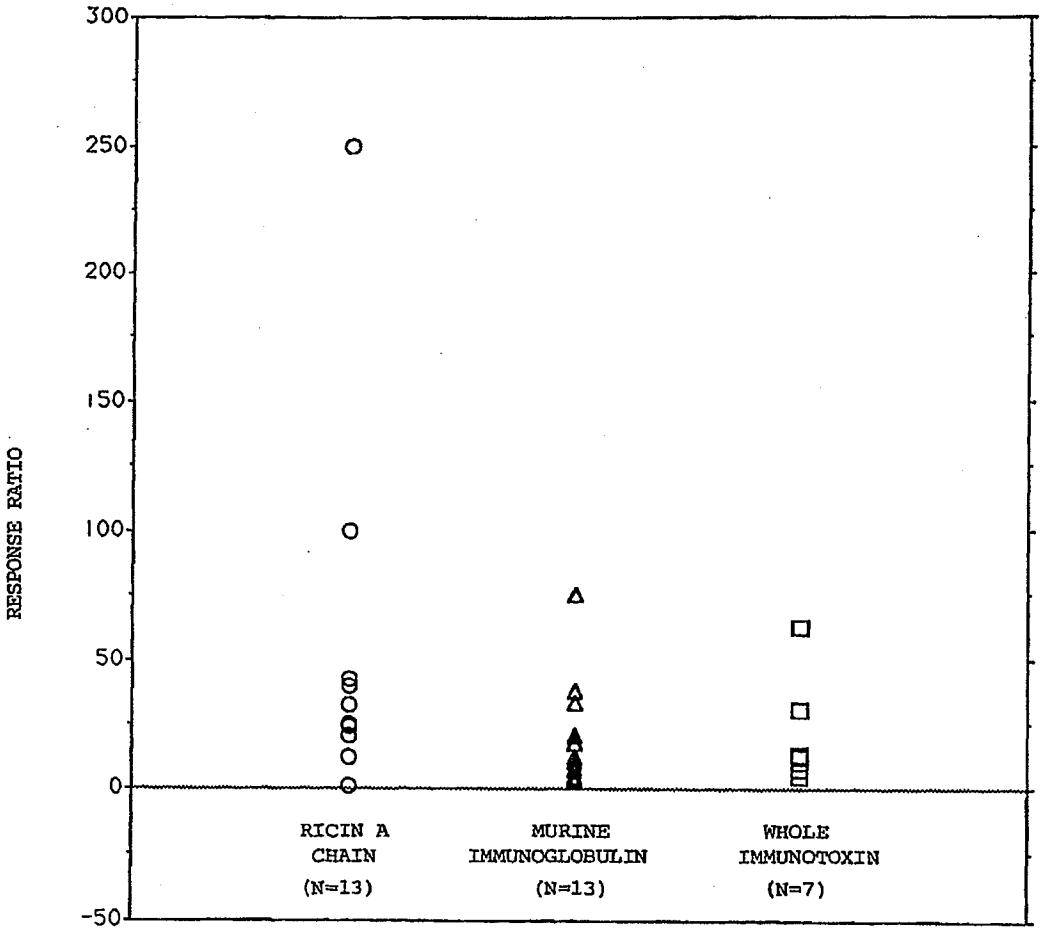

FIG. 1. Response ratios of maximum end-point titers of human antimurine antibodies (HAMA), human antiricin antibodies, and human anti-immunotoxin antibodies.

ies of this combination of immunotoxin plus cyclophosphamide on host antitumor immune responses are needed to confirm the activity seen in our study and to elucidate potential mechanisms of action.

Cyclophosphamide is known to have effects on the cell membrane and it is possible that this resulted in enhanced efficacy of the immunotoxin perhaps by increasing entry of the immunotoxin into the cell or by making the cell more susceptible to the toxic activity of the ricin A chain.

It is noteworthy that a delayed antitumor response occurred in some patients suggesting that mechanisms in addition to direct ricin A chain toxicity may be relevant in the antitumor activity of this immunotoxin. In a number of instances, soft tissue deposits became erythematous and tender in the first week after treatment and then gradually became softer and smaller, mimicking an inflammatory reaction. Furthermore, in one patient with multiple pulmonary metastases, and several with multiple soft tissue nodules, we observed continued, gradual response over a period of several weeks. An excisional biopsy of a responding lymph node in one patient revealed necrosis and small amounts of residual tumor with dense lymphocytic infiltration, 11 months after treatment (Fig. 2A and B). This type of response is similar to the inflammation described at tumor sites during treatment with the R24 antibody.

This time course of response suggests that mechanisms other than direct ricin activity may be relevant in the antitumor activity of this immunotoxin. It has been proposed that monoclonal antibodies induce tumor regressions by directly facilitating antibody- (ADCC) or complement-dependent cellular cytotoxicity (11,12). It is also possible that, as has been demonstrated in a number of preclinical animal studies, monoclonal antibodies interact with host effector cells, stimulating them to recognize and kill tumor cells (13–19). The precise nature of the interactions of the XMMME-001-RTA monoclonal antibody with host effector cells, whether lymphocytes or macrophages, has not yet been defined. More detailed studies perhaps in combination with lymphokines [such as interleukin-2 (IL-2)] and/or monocyte stimulators (γ-interferon) might be conducted in order to elucidate these immune effects.

Also of interest in our study was the pattern of responses. Significant tumor regressions were observed in pulmonary lesions, subcutaneous nodules, and lymph nodes, whereas visceral sites of metastases rarely responded. Antigenic heterogeneity of tumor cells may provide one explanation for this variability in sites of response. It is known that melanoma cells vary widely both at the quantitative level and with respect to qualitative patterns of expressed antigens (20,21). It is possible that a single monoclonal antibody directed against a specific tumor-associated antigen would bind only a portion of and not all tumor cells—thereby producing regressions in only some but not all clinically apparent metastatic deposits. Of interest, in our patients we saw little if any progression in nodules that had initially responded to treatment—progressive disease was almost exclusively noted either in nonresponding metastatic sites—or in the development of new metastases. This pattern of response has been previously noted in earlier clinical trials with immunotoxin XMMME-RA-001, and seems to mirror response patterns observed with chemotherapy and other immunotherapies such as α-interferon, i.e., increased responsiveness of lymph nodes, soft tissue, and pulmonary metastases rather than visceral or bone disease (1,22).

In this protocol, we added cyclophosphamide as an immunosuppressive agent in an effort to abrogate the host antibody response against both the murine antibody and ricin A chain components of the immunotoxin. Cyclophosphamide is known to have selective and potent actions in inhibiting B cell function. The dose and schedule of administration relative to antigenic stimulus are critical in the effect of cyclophosphamide on the immune response. When given in moderate doses prior to antigenic stimulus, immune responses are potentiated (23–25). Treatment following antigenic stimulus may lead to immune suppression (4–8). In this trial, titers and response ratios of human antimurine immunoglobulin antibodies and antiricin antibodies were similar to those seen in earlier studies using XMMME-001-RTA (1). In the dose and schedule employed in this trial, we were unable to demonstrate any suppression of host anti-immunotoxin immune response.

Other investigators working with XMMME-001-RTA have studied the effects of a variety of immunosuppressive regimens on the antibody responses of treated patients. Preliminary data have been reported in abstract form (26,27). Patients receiving aziathioprine/prednisone combinations had more immunosuppression of anti-immunotoxin antibodies than patients receiving moderate dose cyclophosphamide (250 mg/m^2 p.o. $\times$ 5 days) with

A

B

FIG. 2. Excised lymph node 11 months following treatment with XMMME-001-RTA immunoconjugate and cyclophosphamide. (A) 9.7× magnification; (B) 38.6× magnification.

prednisone (100 mg/day × 5 days). Data on the high dose cyclophosphamide (400 mg/m^2 p.o. × 5 days) with prednisone regimen are not yet available. In ¾ patients given cyclophosphamide at 100 mg/m^2 p.o. on days 1–14, antibodies to murine immunoglobulin and ricin A chain components were suppressed. Trials with these agents are ongoing.

In conclusion, we found the combination of the XMME-001-RTA immunotoxin and cyclophosphamide to be safe and more effective than either agent used singly in the treatment of metastatic malignant melanoma. Further in vitro and clinical studies should be directed towards a more detailed definition of the tumoricidal effects of the immunotoxin–cyclophosphamide combination—with specific attention to possible interactions with host effector cells. Administration with IL-2 and lymphokine-activated killer cells, if ADCC is found to be a primary mechanism, or γ-interferon in order to augment monocyte activity, might further enhance the

antitumor effect of this regimen. The role of cyclophosphamide in modulating lymphocyte subpopulations in this regimen requires clarification. More effective means of abrogating host immune response must be explored.

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Exhibit 10

[CANCER RESEARCH 47, 2986–2989, June 1, 1987]

Phase I Study of Recombinant Tumor Necrosis Factor in Cancer Patients[1]

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ABSTRACT

Tumor necrosis factor is a cytokine derived from activated macrophages. This agent is cytostatic and cytolytic against transformed human cell lines *in vitro* and has *in vivo* activity against a variety of murine tumors. We report a clinical study of the pharmacokinetics, toxicity, and biological activity of i.v. and i.m. administered recombinant human tumor necrosis factor (rTNF). Twenty patients with metastatic cancer were given rTNF in doses ranging from 1 to 200 μg/m² by alternating i.m. and i.v. bolus injections with a minimal intervening period of 72 h. Each patient received a maximum of eight treatments given twice weekly over a 4-week period. With i.v. bolus administration, serum concentrations of rTNF were detected by enzyme-linked immunosorbent assay at doses of 25 μg/m² or greater. The clearance of rTNF in the serum was described by a monoexponential equation with a half-life calculated to be 14–18 min. After i.m. administration, serum concentrations of rTNF were consistently detected by enzyme-linked immunosorbent assay at doses of 150 μg/m² or greater. Peak concentrations were observed within 2 h and rTNF was occasionally detected, at the lower limit of sensitivity of the assay, at 24 h postinjection. rTNF was well tolerated clinically in this dose range, and there was evidence of antitumor effect.

INTRODUCTION

TNF[4] was discovered by Carswell *et al.* (1, 2) in 1975. Sera from endotoxin-treated mice, rabbits, or rats that had been sensitized with Bacillus Calmette-Guérin were found to contain an activity that caused hemorrhagic necrosis of rodent tumors. Subsequent experiments *in vitro* have shown that leukocytes produce at least two distinct cytotoxic factors by immune effector cells; activated macrophages produce TNF-α, whereas mitogen-stimulated lymphocytes produce TNF-β (previously called lymphotoxin) (3, 4). The genes encoding both TNF-α and TNF-β have recently been sequenced and cloned and have been shown to have an approximate 28% amino acid homology (5, 6). Both cytokines are known to have cytostatic and cytotoxic effects *in vitro* against a wide range of human tumor cells but have no such effects against normal human fibroblasts (1, 2, 8). Antitumor effects have been demonstrated in both syngeneic murine tumors and human tumor xenografts in nude mice (9). In addition, marked synergistic antiproliferative activity has been demonstrated with TNF-α (or TNF-β) and γ-interferon against both murine and human tumor cell lines *in vitro* (10, 11). Although the exact mechanism by which TNF exerts its antitumor activity is unknown, it has been hypothesized that TNF must first bind to a cell surface receptor, be internalized, and then perhaps trigger the release of lysosomal enzymes that lead to lysis of the target cell.

The availability of TNF produced through recombinant DNA technology has enabled the exploration of the therapeutic potential of TNF as an anticancer agent in human clinical trials. We report the results of a multiple escalating dose Phase I clinical trial of rTNF. This biological agent is well tolerated when given as a single i.v. or i.m. injection, and it has biological activity in cancer patients.

MATERIALS AND METHODS

Preparation of rTNF. The molecular cloning and protein characterization and purification of rTNF were performed by Genentech, Inc. (South San Francisco, CA) and have been described previously (3–6). The rTNF produced in *Escherichia coli* is nonglycosylated and has a molecular weight of approximately 17,000. rTNF is purified to more than 99% as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and has a specific activity of approximately 4×10^7 units/mg of protein as defined by the lysis of actinomycin D-treated mouse L929 cells (12). Sterility, general safety, and purity studies meet Office of Biologics, Food and Drug Administration standards.

Patient Selection and Study Design. Patients with histopathological confirmation of disseminated cancer who had not received antitumor therapy for at least four weeks were entered on the study. Eligibility criteria included a performance status of ≥50% (Karnofsky scale) (13), life expectancy of at least 3 months, preserved hepatic (bilirubin, <1.5 mg/dl), renal (creatinine, <2.0 mg/dl), and hematological (granulocytes, ≥1,500/mm³; platelet count, ≥100,000/mm³) function. Informed consent was obtained in accordance with institutional policy. Patients were observed in the hospital for 36 h after each dose.

Table 1 describes the treatment plan. Two patients were entered sequentially at DOSE LEVELS 1 through 10 and received twice weekly treatment for 4 weeks. The first two patients received 5 μg/m² i.m. and 1 μg/m² i.v. (LEVEL 1), the next two patients received 10 μg/m² i.m. and 2 μg/m² i.v. (LEVEL 2), etc. A minimum interval of 72 h was maintained between doses. Each patient received a maximum of four sequential dose escalations weekly over a 4-week period. This study design was used to observe single dose tolerance and pharmacokinetics over a wide dose range. It was recognized that the maximum tolerated dose for Phase II trials could not be determined due to the possible occurrence of cumulative toxicity from intrapatient dose escalations. Fixed multiple dose Phase I trials to achieve this objective are now in progress.

Patients were monitored daily. All constitutional symptoms were recorded and classified as minimal (Grade I), moderate (Grade II), severe (Grade III), and life threatening (Grade IV). Vital signs were recorded before injections and at 20 min and at 1, 2, 4, 6, 8, 12, 18, and 24 h after each injection. A history and physical examination were done before the initial dose and twice weekly (preinjection) thereafter. Patients were weighed daily. An electrocardiogram and chest x-ray were done before the study and after the final dose. A complete blood count, coagulation profile, and determination of serum electrolytes were done prior to each dose and at 4 and 24 h after each dose. A serum chemistry profile (including renal and liver function tests and triglyceride and cholesterol levels), urinalyses, and reticulocyte counts were obtained before and 24 h after each dose. Tumor size was evaluated by physical examination and appropriate radiological studies and scans, as well as by tumor markers. The criteria for antitumor response have been described previously (14).

Pharmacokinetic Studies. Venous blood samples were collected before injection and at 20 min, 40 min, and 1, 1.5, 2, 4, 6, 8, 12, 18, and 24 h following i.m. doses and at 5, 10, 20, and 40 min and 1, 1.5, 2, 4, 6, 8, 12, 18, and 24 h after i.v. doses. Blood samples were centrifuged and the serum was decanted and stored at ≤−20°C until analysis.

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[4] The abbreviations used are: TNF, tumor necrosis factor; rTNF, recombinant tumor necrosis factor; ELISA, enzyme-linked immunosorbent assay.

Table 1 *rTNF dose levels*

Dose levels	Dose ($\mu g/m^2$) i.m.	i.v.
1	5	1
2	10	2
3	15	5
4	25	10
5	35	15
6	50	25
7	65	35
8	80	50
9	100	65
10	125	80
11	150	100
12	175	125
13	200	150

Serum rTNF titers were measured by ELISA at Genentech, Inc. A solid-phase ELISA was performed using a sandwich technique with two polyclonal antibodies to rTNF, one of which was conjugated to a horseradish peroxidase label. The ELISA assay can detect rTNF reliably at 100 to 2800 pg/m. In addition, a bioassay (L-M cell cytotoxicity with actinomycin D added) (15) was performed on specimens that were positive in the ELISA.

The mean serum concentrations at each time point for all patients at a given dose were calculated. Individual serum rTNF disposition curves were constructed and nonlinear regression analyses were performed for each patient. Serum concentration half-lives, volumes of distribution, and the area under the concentration curve were then calculated for each patient individually in standard fashion (16).

The presence of antibodies to rTNF was determined on blood samples obtained before the study and 3 to 4 and 28 to 30 days after completion of the study.

Statistics. The Wilcoxon signed rank test was used for the determination of statistical significance between pre- and post-laboratory values.

RESULTS

Twenty patients with disseminated cancer were treated, 7 men and 13 women ranging in age from 26 to 75 years, with a median age of 54 years. Five patients had colon cancer, 2 had renal cell carcinoma, 4 had breast cancer, 2 had pancreatic cancer, 3 had multiple myeloma, and 1 each had nodular poorly differentiated lymphoma, melanoma, adenocarcinoma of the gastroesophageal junction, and Hodgkin's disease. The patients with multiple myeloma and pancreatic cancer had received no prior therapy, whereas all other patients had been treated with radiation therapy, chemotherapy, and/or immunotherapy. Two of the 20 patients were removed from the study after they received 1 dose of rTNF because they did not meet the eligibility criteria.

The most common clinical side effects associated with rTNF therapy are described in Table 2. The side effects were similar following i.m. and i.v. administration. Although fever and chills occurred in almost every patient at all dose levels, they were not dose limiting and generally resolved within 12–24 h. Headaches were common but were easily treated with acetaminophen. Although mild soreness at the i.m. injection site was common, no ulceration occurred. The onset of muscle soreness was at 6–12 h and the duration was 24–72 h. With increased dose, muscle soreness became more severe but was never dose limiting. Further, there were no consistent adverse hemodynamic changes. Significant hypotension was not observed. No patient experienced a significant (≥10%) weight loss. Respiratory insufficiency and hepatic dysfunction occurred following two doses of rTNF (12 $\mu g/m^2$ total dose) in a patient with

Table 2 *Adverse clinical effects of rTNF*

	1–15 $\mu g/m^2$		25–65 $\mu g/m^2$		80–200 $\mu g/m^2$	
	i.m.	i.v.	i.m.	i.v.	i.m.	i.v.
No. of patients	7	10	13	13	10	5
No. of evaluable treatments	14	25	29	28	28	13
Adverse effects						
Fever	14[a] (100)[b]	24 (96)	29 (100)	27 (96)	28 (100)	13 (100)
Chills	9 (64)	20 (80)	22 (76)	24 (80)	23 (82)	11 (85)
Headaches	3 (21)	8 (32)	13 (45)	7 (25)	15 (54)	10 (77)
Fatigue	3 (21)	5 (20)	9 (31)	9 (32)	11 (40)	8 (62)
Anorexia	3 (21)	8 (32)	8 (28)	3 (11)	11 (40)	6 (46)
Nausea	2 (14)	3 (12)	3 (10)	2 (7)	8 (29)	7 (54)
Vomiting	2 (14)	1 (4)	4 (14)	2 (7)	2 (7)	3 (23)
Diarrhea	1 (7)	0 (0)	2 (7)	1 (4)	2 (7)	1 (8)
Dizziness	3 (21)	1 (4)	4 (14)	2 (7)	6 (21)	1 (8)
Myalgia	0 (0)	1 (4)	3 (10)	1 (4)	4 (14)	1 (8)

[a] Values represent the number of treatments associated with adverse effects.
[b] Numbers in parentheses, percentage of patients experiencing adverse effects.

breast cancer and extensive hepatic and pulmonary lymphangitic metastases. This patient died 7 days following the last rTNF injection. A second patient with colon cancer and pulmonary metastases was removed from the study after experiencing transient respiratory insufficiency. A full recovery to the pretreatment clinical condition followed. The onset of acute respiratory insufficiency in both patients occurred within 6–12 h after receiving rTNF.

The effect of rTNF on platelet, granulocyte, and lymphocyte counts is presented in Table 3. Twenty-four h after i.m. administration of rTNF, there was a statistically significant, although clinically unimportant, decrease in platelet count. This effect was neither cumulative nor dose dependent. The platelet counts recovered by 48–72 h. In contrast, 24 h after i.v. administration of rTNF, no significant decrease in platelet counts occurred. Four h after the administration of either i.m. or i.v. rTNF, there was a significant decrease in the absolute lymphocyte count and an increase in the granulocyte count (Table 3). By 24 h the absolute granulocytes had returned to normal while the absolute lymphocytes had not. With respect to hemoglobin, among the 16 patients who completed 1 month of therapy there was a significant decrease from pretreatment hemoglobin levels that was not dependent on the cumulative TNF dose (Table 4). The drop was significantly below the level that we observed after 1 month of therapy in our γ-interferon trial which had similar phlebotomy requirements (17). rTNF also affected certain biochemical parameters. After patients completed the 8 doses of rTNF or 1 month of therapy, there was a statistically significant increase in serum triglyceride and a significant decrease in cholesterol (Table 5) and serum albumin levels (data not shown). There was no laboratory evidence of renal or hepatic toxicity.

Table 6 is a summary of the pharmacokinetic parameters calculated from the ELISA-detectable concentrations of rTNF in the serum of patients receiving i.v. bolus doses of rTNF ranging from 25 to 100 $\mu g/m^2$. The half-life and dose-corrected area under the curve for rTNF given by i.v. bolus administration appear to increase with increasing dose (Fig. 1; Table 6). The apparent volume of distribution (V_d) was 66 liters at the 25-$\mu g/m^2$ dose and decreased to 12 liters at the 100-$\mu g/m^2$ dose. After i.v. bolus injection, the clearance of ELISA-detectable material closely fit (r^2>0.95) an open one-compartment mathematical model (Fig. 1). Moreover, determinations of rTNF activity in the serum following i.v. bolus administration using the L-M cell cytotoxicity bioassay correlated with ELISA measurements (data not shown). In contrast to the i.v. bolus route, i.m. administered rTNF was not consistently detected in patients'

Table 3 *Changes in hematological indices 4 and 24 h after rTNF administration*

Hematological index	Route of administration	No. of treatments	Pretreatment mean ± SE ($\times 10^3$ cells/mm^3)	4 h posttreatment mean ± SE ($\times 10^3$ cells/mm^3)	24 h posttreatment mean ± SE ($\times 10^3$ cells/mm^3)	*P*
Platelets	i.m.	59	343 ± 21		292 ± 19	<0.001
	i.v.	60	322 ± 20		332 ± 22	NS[a]
Granulocytes	i.m.	59	4.8 ± 0.29	7.03 ± 0.43		<0.001
			4.8 ± 0.29		4.8 ± 0.23	NS
	i.v.	60	4.36 ± 0.28	7.48 ± 0.39		<0.001
			4.36 ± 0.28		4.57 ± 0.27	NS
Lympho-cytes	i.m.	59	1.05 ± 0.1	0.56 ± 0.05		0.001
			1.05 ± 0.1		0.79 ± 0.08	0.001
	i.v.	60	1.0 ± 0.07	0.56 ± 0.05		<0.001
			1.0 ± 0.07		1.12 ± 0.07	0.05

[a] NS, not significant.

Table 4 *Change in hemoglobin after 1 month of rTNF therapy*

Cumulative rTNF dose (μg/m^2)	No. of patients	Pretreatment hemoglobin (g/dl) median (range)	Posttreatment hemoglobin (g/dl) median (range)	*P*
73–180	5	11.1 (10.7–15.2)	9.7 (6.8–9.8)	<0.05
255–470	6	12.4 (10.3–14.0)	8.9 (7.0–11.5)	<0.01
595–1105	5	11.6 (10.3–14.3)	9.3 (8.5–12.1)	<0.001

Table 5 *Change in lipids after 1 month of rTNF therapy*

Lipids	No. of patients	Pretreatment median (range) (mg/dl)	Posttreatment median (range) (mg/dl)	*P*
Triglyceride	16	119 (59–296)	125 (68–384)	0.025
Cholesterol	16	164 (100–284)	142.5 (88–231)	<0.001

Table 6 *Serum pharmacokinetics of rTNF by ELISA after i.v. bolus administration*

Dose (μg/m^2)	No. of studies	Half-life[a] (min ± SE)	Apparent V_d[b] (liters ± SE)	AUC (ng/ml × min)
25	3	15.9 ± 3.6	66 ± 30	10.5 ± 2.7
35	5	13.9 ± 1	31.3 ± 5	19.7 ± 5.3
50	4	16 ± 2	13.4 ± 1.1	89.6 ± 13.9
65	5	18 ± 0.4	17.7 ± 4	114.6 ± 26.5
100	3	17 ± 2	12 ± 4	223.8 ± 69

[a] Values expressed as means.
[b] V_d, volume of distribution; AUC, area under the concentration curve.

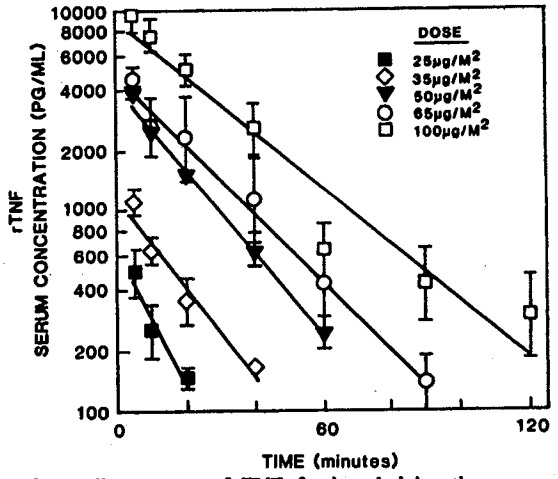

Fig. 1. Serum disappearance of rTNF after i.v. administration as measured by ELISA. *Symbols* represent the mean of blood levels for each dose for all patients at that dose. *Standard error bars* are shown unless insufficient data points were available.

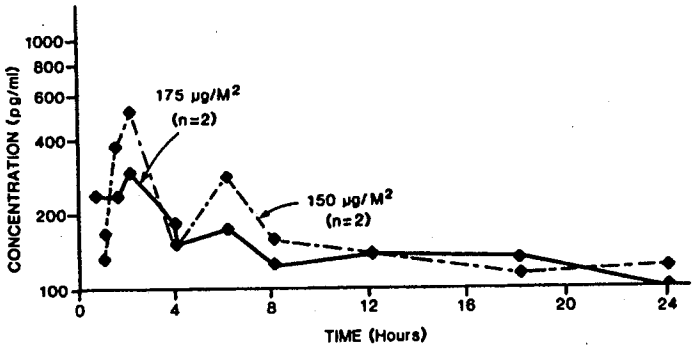

Fig. 2. Serum disappearance of rTNF after i.m. administration as measured by ELISA. ◆, mean of the serum levels for each dose for all patients at that dose.

sera by ELISA until doses reached 150 μg/m^2 or greater. Peak serum levels were usually obtained with 2 h and ELISA-positive material occasionally persisted for 24 h after the injection (Fig. 2).

None of the patients had circulating antibodies to TNF prior to the study, nor did any patient develop antibodies by the completion of the study.

Among the 16 evaluable patients that completed 4 weeks of therapy, there was evidence for antitumor effect in 2 patients. There was complete regression of a 5- x 5-cm neck lesion but no demonstrable effect on extensive retroperitoneal disease in a patient with renal cell carcinoma; there was resolution of malignant ascites in another patient with metastatic colon cancer.

DISCUSSION

Mononuclear cells secrete a variety of cytokines in response to foreign antigens: interleukins (18); interferons (19); and TNFs (3–6). TNF has a wide range of biological properties (for review see Ref. 20). Since the discovery that this factor was able to elicit hemorrhagic necrosis of tumors *in vivo* (1, 2), considerable effort has gone into the large scale production of TNF by recombinant DNA methodology. This report describes the pharmacokinetics, biological effects, single dose tolerance, and toxicity of rTNF in cancer patients.

The clearance of rTNF as measured by ELISA following i.v. bolus administration was monoexponential, suggesting an open one-compartment mathematical model (Fig. 1). This appears to be similar to the pharmacokinetics of other recombinant lymphokines (17, 21). In contrast to the i.v. route of administration, i.m.-administered rTNF occasionally resulted in sustained concentrations of rTNF in the serum. Because of the

small number of measurements for each patient studied, pharmacokinetic calculations were not possible after i.m. administration.

Fever and chills were nearly universal following rTNF therapy irrespective of the route of administration. The mechanism of the pyrogenic effect of rTNF is not known but may involve a direct effect on the hypothalamic thermoregulatory centers or an induction of the biosynthesis of another monokine, interleukin 1 (22). An inflammatory reaction occurred at the i.m. injection site but was not dose limiting. Significant hypotension and weight loss were not observed. Respiratory insufficiency occurred in two patients but in both cases these events were attributed to the patient's underlying tumor.

TNF administered in the dose schedule used in our study did not result in granulocytopenia. Since TNF is known to inhibit granulocyte-macrophage progenitor cells *in vitro* (23),[5] perhaps higher doses and/or a more prolonged exposure to TNF will be necessary to achieve this effect *in vivo*. The fact that platelet counts were suppressed only after TNF was administered by the i.m. but not the i.v. route suggests that prolonged exposure to circulating levels of TNF may have accounted for the suppression. The effects we noted on peripheral WBC after 4 h of rTNF administration in all likelihood were a result of an acute phase reaction which is in part known to be mediated by TNF (20). TNF is also known to inhibit erythropoiesis (24). The significant drop in hemoglobin noted in our study population may indicate that this inhibition occurred *in vivo*. TNF is also thought to inhibit lipoprotein lipase activity (25, 26). The decrease in cholesterol and elevated triglyceride levels that we noted may have been a consequence of this inhibition.

In conclusion, we have shown that rTNF is well-tolerated when administered in single doses up to 200 $\mu g/m^2$, can be detected in the serum by ELISA following i.v. and i.m. injection, and shows evidence of antitumor effects in this dose range.

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[5] M. Beran, B. Anderson, K. B. McCredie, and J. U. Gutterman. Tumor necrosis factor and human hematopoiesis *in vitro*, submitted for publication.

Exhibit 11

THE DESIGN OF CYTOTOXIC-AGENT-ANTIBODY CONJUGATES

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ABBREVIATIONS

AFP = Alpha-fetoprotein
ALL = Acute lymphocytic leukemia
ARA-C = Cytosine-1-beta-D-arabinoside
BSA = Bovine serum albumin
CALLA = Common acute lymphoblastic leukemia antigen
CEA = Carcinoembryonic antigen
CLL = Chronic lymphocytic leukemia
CPK = Creatine kinase
DHFR = Dihydrofolate reductase
DTT = Dithiothreitol
ECDI = 1-Ethyl-3-(3′-dimethylaminopropyl)carbodiimide
EGF = Epidermal growth factor
FUR = 5′-Fluorouridine
hCG = Human chorionic gonadotropin
hPL = Placental lactogen (chorionic somatomammotropin)
HSA = Human serum albumin
HTLV = Human T-cell leukemia virus
I_{50} = Concentration giving 50% inhibition
Ig = Immunoglobulin
LH = Luteinizing hormone
MAA = Melanoma-associated antigen
MAB = Monoclonal antibody
MTX = Methotrexate
NHS = *N*-hydroxysuccinimide
PAGE = Polyacrylamide gel electrophoresis
PAP = Prostatic acid phosphatase
PBS = 0.01 *M* Sodium phosphate, containing 0.145 *M* sodium chloride

PDT = Pyridyldithio
pGA = Polyglutamic acid
PP5 = Placental protein 5
SDS = Sodium dodecylsulfate
SMBE = *N*-succinimidyl m-(*N*-maleimido)-benzoate
SMBU = *N*-succinimidyl m-(*N*-maleimido)-butyrate
SPDP = *N*-succinimidyl-3-(2-pyridyldithio)-propionate
SPI = Pregnancy-specific glycoprotein
TAA = Tumor-associated antigen
TATA = Tumor-associated transplantation antigen
TLC = Thin-layer chromatography

I. INTRODUCTION

In spite of remarkable advances in cancer chemotherapy, most chemotherapeutic agents and ionizing radiations currently in use have a low therapeutic index,[1] i.e., they damage all proliferating cells without discriminating between neoplastic and normal tissues, including such vital tissues as bone marrow, lymphoid tissue, gastrointestinal and genitourinary epithelium, etc.[2] The search for methods of rendering agents tumor specific has led to the experimental use of a wide array of carriers with various degrees of tumor-specificity.[3,4] Nonspecific carriers include cells, liposomes, and polymeric drug formations.[3,4]

If cancer cells have distinctive molecules on their surface and they are accessible, then one can use specific ligands to carry cytotoxic agents selectively to the target tumor, e.g., hormones,[5-7] transferrin,[8] lipoproteins,[9] lectins,[10,11] and antibodies.[12] The availability of MAB against a wide variety of TAA has now led to the accumulation of a substantial body of evidence in support of antibody-mediated drug targeting. Ghose and Blair evaluated the status and prospects for treatment of cancer in 1978[12] and later updated this, emphasizing their own experience in the field.[13,14] They have outlined methods that are useful in evaluating the antitumor activity of drug-antibody conjugates in vitro and in clinically relevant in vivo tumor models.[13] They have also reviewed the evidence for the localization of both polyclonal and monoclonal anti-TAA antibodies in target tumors in vivo and the feasibility of using labeled antibodies for the detection and possible therapy of cancer.[15-17] The pioneering studies of Landsteiner on the linkage of small haptenic moieties to carrier proteins and the wide application of this approach in the production of antibodies for radioimmunoassay have made available novel and milder methods of linkage of chemotherapeutic agents and toxins to Ig. Ghose and co-workers have outlined the strategy of linkage and have described those methods that have been used or are potentially useful for the production of biologically active Ig-linked anticancer agents.[13,14,18]

This review will be confined to antibody-mediated drug and toxin delivery systems as applied to cancer therapy. The evidence for and against the basic tenets of this approach will be evaluated. That is, are there antigenic moieties on tumor cells that can be therapeutically exploited? Do antibodies against these antigens selectively localize in target tumors in vivo? Can antibodies transport an active cytotoxic agent to the site of action of the agent, especially when such sites are intracellular? Liposomes will be dealt with only when they have been coated with an anti-TAA antibody to target packaged drugs. The widespread (but still experimental) use of antibody-linked radionuclides for tumor detection will not be considered. A compilation of selected abstracts on tumor localization of radiolabeled antibodies is available.[19]

II. TUMOR MARKERS AND TUMOR-ASSOCIATED ANTIGENS AS TARGETS FOR ANTIBODY-MEDIATED DELIVERY OF CYTOTOXIC AGENTS

There is evidence that chemically or virus-induced experimental tumors may acquire antigenic moieties that are not demonstrable in normal homologous tissues.[20,21] TATA induced by chemicals such as methylcholanthrene are highly immunogenic, stable, and heritable in a given tumor line. However, an important feature is their polymorphism.[22] For example, each methylcholanthrene-induced tumor appears to express a distinctive TATA, even when they are at different sites in the same animal. In certain cases, individual clones of cells from the same tumor have been found to express one or more distinct non-cross-reacting TATA.[23]

A given tumor cell may express more than one TATA. However, recent results[24] suggest that at least some of the TATA cross-react among different tumors and the number of different TATA is probably not large. In the context of the present review, an interesting feature of the TATA of chemically induced mouse (but not of rat) tumors is their inability to induce a humoral response in the host. However, such TATA of mice (and other species) may elicit an antibody response in a foreign species and thus provide polyclonal antibodies and MAB for targeting. The TATA of chemically induced rodent tumors differ from oncogenic virus-induced TATA in that tumors induced by the same or closely related viruses share a common TATA.

Distinctive antigenic moieties like the TATA of chemically induced rodent tumors have not been found on human tumor cells. In the context of antigenicity, Weiss[20] has described two categories of "spontaneous" human tumors (i.e., tumors that arise without experimental manipulation irrespective of their etiological factors), namely those that arise in (1) severely immunosuppressed individuals and (2) immunocompetent individuals. Tumors induced by oncogenic viruses predominate in the first group. A variety of inciting agents, e.g., cigarette smoke, ultraviolet (UV) irradiation, asbestos, and probably some viruses as well, may be implicated in the second group of tumors. Virus-induced tumor formation and its progression may involve the activation of one or a series of oncogenes leading to either amplified synthesis of a normal product or the expression of an altered product which may be immunogenic.[25-27]

In addition to the very small number of human tumors of possible viral etiology (e.g., Burkitt's lymphoma, nasopharyngeal carcinoma, and T-cell leukemia) in which various virus-associated antigens have been detected in the transformed cells,[28] there is also evidence that many human tumors synthesize substances that are either absent from or are produced in extremely limited amounts by the homologous normal tissue. These substances are termed tumor markers or TAA when they either provoke or serve as targets of immunologic reactivity. (In the present context, antibody response is more important than cell-mediated immunity.) Many human TAA are associated with retrodifferentiation and are the products of reexpressed fetal genes, i.e., the so-called oncofetal antigens. Some, such as CEA and AFP, may be present in normal serum and tissues in minute amounts. However, a variety of conditions, e.g., hyperplasia, may substantially increase the production of these or cross-reacting antigens. Furthermore, with the availability of panels of MAB directed against different epitopes of a given TAA, cross-reactivity of many TAA with normal cellular components has been observed (see later). There may also be aberrant expression of various differentiation genes in cells during neoplastic transformation. For example, many human tumors may secrete one or another polypeptide hormone even though the homologous normal tissues might not. Neoplasms that secrete a hormone, aberrantly or otherwise, can be excellent targets of agents linked to antibodies against that hormone, provided that the

possible loss of the hormone-producing normal cells can be compensated for by appropriate replacement therapy.

In the same way, antibodies against other normal tissue antigens (indigenous or aberrant) that are expressed in a tumor cell population can be used for targeting cytotoxic agents if the normal cell population expressing the antigen is not vital for survival. For example, prostate-specific acid phosphatase, if secreted by a prostatic carcinoma, can be a useful target provided that the antibody against it does not cross-react with the acid phosphatase in such vital tissues as renal tubular epithelium. Casein and lactalbumin, normally synthesized by mammary epithelium during pregnancy, can be aberrantly expressed in mammary carcinomas and can thus be useful targets. B-cell lymphoma/leukemias are usually monoclonal and therefore express Ig of a particular idiotype. Antibodies directed against the idiotype of the Ig associated with such monoclonal B-cell neoplasms are uniquely specific and have been used for drug-targeting and/or serotherapy of experimental and human tumors.[29] Altered activity of one or more glycosyl transferases observed in transformed cells can lead to the incorporation of aberrant sugar residues into cell surface-associated glycolipids or glycoproteins and thus change the antigenic profile of tumor cell surface components. Finally, the alloantigen(s) in choriocarcinomas (which are of fetal origin) may be regarded as a TAA.

Since the introduction of hybridoma technology, ever-increasing numbers of reports are appearing on the production of "specific" MAB against human tumors. The tumor specificity of most remains to be conclusively established. In the usual practice for characterization, the product of a given MAB-producing clone is screened against target tumor cells and a panel of normal tissues. Such screening procedures usually fail to detect antigen(s) in minor normal cell populations (especially when not easily accessible) and normal antigens that are expressed only at a certain stage of differentiation or a certain phase of the cell cycle. For example, MAB-defined Ca-1 antigen, once thought to be specific for human epithelial malignancies, has now been found in a number of normal tissues (see later). In the following section, those human TAA that have potential for use in antibody-mediated drug-targeting will be discussed.

A. Pan-Cancer Antigen(s)

An antigen that is present in all or most malignant tumors, irrespective of their histogenesis and status of differentiation, will be of great help in the diagnosis and treatment of cancer. Extensive efforts have been made to obtain and characterize such pan-cancer antigens; however, few have withstood careful scrutiny of the claimed specificity. For example, the polypeptide antigen(s) claimed to be present in different types of carcinomas[30] has been found in many normal tissues, especially in non-keratinizing epithelium and mesothelium and in normal serum.[31-33] The MAB-Ca-1-defined antigen designated by Ashall et al.[34] as Ca was claimed to be expressed by the majority of malignant tumors and only in the normal epithelial lining of fallopian tubes and bladder. However, subsequent studies have revealed reactivity of the Ca-1 MAB with a variety of normal tissues (for example, see Woodhouse et al.[35]). Ca 19-9, another MAB-defined antigen claimed to be a marker of several different types of cancer (e.g., colorectal, gastric, and pancreatic carcinomas), has been demonstrated to be chemically identical with the sialylated Lewis a^- determinant of a monoganglioside and a mucin. It has been found in large amounts in the seminal plasma of healthy donors.[36] Very small amounts of a malignancy-associated sialylated glycoprotein were isolated by Bolmer and Davidson[37] from the perchloric acid-soluble fraction of serum from patients with various types of cancer. This "cancer glycoprotein" had a high affinity for wheat germ agglutinin and *Ricinus communis* agglutinin. It was claimed to be shed from tumor cell surfaces, to be not one of the major acute phase reactant glycoproteins associated with malignancy, and distinguishable from CEA and AFP by its molecular

weight and chromatographic behavior. However, whether this glycoprotein is indeed present on the cancer cell surface and its tumor specificity remain to be ascertained. Oncomodulin is a parvalbumin-like calcium-binding protein found in 85% of rodent and human tumors tested but is not detectable in normal adult tissues and therefore has potential as a tumor marker.[38] Methods for immunocytochemical detection[38] and RIA[39] of oncomodulin have been developed. These may be of help in studying its expression in various human tumors and normal tissues. Malignin and other cancer recognins are low molecular weight polypeptides which are produced by a wide variety of tumors. It has been suggested that malignin may be a general transformation protein and may thus be a useful pan-cancer antigen. It is antigenic and induces the production of a specific antibody in the tumor host. An MAB against malignin has also been produced,[40] but its tumor specificity and diagnostic and therapeutic usefulness need further investigation.

Thus, the tumor specificity of most of the pan-cancer antigens is open to question. However, it should be pointed out that most of the anti-tumor MAB usually react with more than one and sometimes a large number of different histological types of tumors. For example, MAB produced against a 48-kdalton peptide antigen of ovarian cancer reacted with 90% of ovarian cancer and 60% of other human tumors, both benign and malignant, but not with any normal adult tissue.[41]

B. Glycoconjugates in Cancer Cells

Many of the behavioral characteristics of cancer cells, i.e., decreased adhesiveness, loss of contact inhibition, invasiveness, increased growth, and immortalization, may be attributed to changes in cell surface components, especially carbohydrates.[42-46] Differences in monosaccharide content and linkage, sequence, branching, and configuration in cell surface-bound carbohydrates have been observed in a wide variety of experimental and human tumors compared to their normal tissue of origin and may offer one basis for the multitude of TAA specificities.[45-47] Details of the changes during malignant transformation can be found elsewhere.[45,48] Here, the emergence of unique carbohydrate moieties in tumors that might be exploited for drug-targeting will be considered only briefly.

Hakomori[45] has tentatively listed ten types of chemical changes in the carbohydrate moieties of glycolipids and surface glycoproteins on tumor cells compared to those on homologous normal cells. These changes are brought about either by blocked synthesis of normally existing carbohydrate chains or by neosynthesis as a result of activation of glycosyl transferases that are virtually absent from normal cells. The most commonly observed change in surface-bound glycoproteins in cancer cells has been the appearance of relatively high molecular weight glycoproteins replacing smaller ones, i.e., the Warren-Blick-Buck phenomenon.[49] Its structural basis has been attributed to "increased branching at the mannose units of complex oligosaccharides and/or increased sialylation of carbohydrate moieties".[45,48] Several MAB against human TAA are apparently directed against sialosyl residues of a densely glycosylated region of sialosyl-oligosaccharide-containing glycoproteins.[34,50-53] The differences from glycopeptides of normal homologous cells usually (but not always) disappear after treating high molecular weight glycoconjugates from cancer cells with sialidase.[48] From these and similar studies, it appears that the branching enzyme, *N*-acetyl glucosamine transferase, may play a crucial role.

There is no firm evidence that even tumors of a given histogenesis contain a common tumor-associated glycoconjugate or that the tumor cell surface contains unique carbohydrate sequences that are absent from all normal cells. Differences so far found are mainly quantitative, involving several membrane constituents. However, an increasing number of reports deal with the production of MAB that react with tumor-associ-

ated glycoconjugates that are present in all or a proportion of one or more given histological types of tumor and are fairly (though not absolutely) tumor specific. Hakomori[45] and Feizi[46] have also listed TAA, whose carbohydrate moieties have been defined by either classical chemical-immunological analysis or by use of MAB. The analysis of various human tumors has revealed the cessation of synthesis of long chain glycolipids leading to accumulation of immunogenic precursors that could be defined by MAB, e.g., G_{D3} in human melanoma[54,55] and acute nonlymphocytic leukemia,[56] G_{D2} in neuroectodermal tumors,[57] Gg3 in Hodgkin's lymphoma,[58] and Gb_3 in Burkitt's lymphoma.[59] Furthermore, MAB with highly restricted specificity have been obtained against a variety of fucolipids and fucogangliosides that have been observed to accumulate in various human cancers, especially those derived from endodermal epithelium, e.g., the gastrointestinal tract,[60-64] lung,[45] and mammary gland.[65] These antigens may result from activation of aberrant fucosyl or sialosyl transferases and may thus be regarded as the products of neosynthesis.

Another mechanism underlying the emergence of carbohydrate-containing neoantigens may be incomplete or blocked synthesis of glycolipids and glycoproteins. Precursor glycoconjugates, especially glycolipids, may accumulate in cancer cells as a result of blocked synthesis of more complex glycolipids. Reduction in size has been observed, especially in glycolipids,[48] although in transformed cells, the predominant change in cell surface-bound carbohydrates is increased size. New carbohydrate moieties appear in some tumors, whereas carbohydrate moieties cease to be synthesized in others. Decreased sialylation of tumor glycoproteins is also a fairly common observation.[66]

Alterations in the steric arrangement of the carbohydrate and other immunogenic moieties of cell surface-associated glycoconjugates may also play a role in the expression of TAA. Factors that are important in this context include: (1) concentration of epitopes per unit area of the cell surface and the uniformity of their distribution, i.e., the state of cluster; (2) contiguity of epitopes to masking moieties, e.g., other glycolipids and proteins; and (3) orientation and steric stability of the epitopes which will depend upon ceramide composition, cholesterol content, degree of unsaturation of the phospholipid acyl chains, and the protein/lipid ratio in the cell membrane.[44,45,67] Such TAA may be exploited for drug-targeting.

C. Blood Group Antigens as Tumor Markers

The alterations in the expression of blood group glycoprotein and glycolipid antigens in malignancy have been extensively reviewed by Hakomori[68] and Hakomori and Kobata.[69] These changes include: (1) deletion of host blood group antigen, (2) accumulation of precursors, and (3) emergence of new blood group determinants foreign to the host. Only changes under (2) and (3) above are pertinent to this review.

1. Precursor Accumulation

The core glycolipid structure that is the precursor to A, B, O blood group substances has been found at elevated levels in gastric and colonic adenocarcinomas.[70] The M, N blood group precursor antigens T and Tn have been found at elevated levels in about 90% of carcinoma tissues including breast, colon, and gastric carcinoma.[71] Most healthy human tissues, including fetuses, contain T and Tn antigens that are masked by sialic acid covalently linked carbohydrates and/or by tertiary structures and therefore are not immunoreactive. In contrast, both primary and metastatic adenocarcinomas and some squamous cell carcinomas contain immunoreactive forms of T and Tn antigens. The "unmasking" of these two antigens in tumor tissue may be brought about by the blocked synthesis of N or M antigens, increased activity of T and Tn synthesizing transferases, or excessive enzymatic desialylation in cancer cells. The desialylated antigens are treated as foreign by the tumor host and lead to elicitation of a

cell-mediated immune response.[71] Naturally occurring anti-T and anti-Tn antibodies are found in the serum of all humans, probably as a result of antigen stimulation from intestinal flora. This is in contrast to the lack of immunogenicity of oncofetal antigens. Circulating anti-T antibody binds to the unmasked T antigen on the surface of carcinoma cells and sheds cell surface components that contain T antigen. Human anti-T antibody in the presence of complement kills carcinoma cells in vitro. Apart from antibodies, T and Tn antigens bind to peanut lectin. Radioiodinated peanut lectin[72] and non-IgM MAB against T and Tn antigens[71] have been successfully used for the detection of T- or Tn-containing carcinoma xenografts in mice, a species that has no or little preexisting anti-T and anti-Tn antibodies.[71]

2. New Blood Group Determinants

Early studies by Hakomori[68] and other groups have revealed that: (1) tumor tissues may delete A and B determinants due to blocked activity of A and B transferases; (2) the type 2 chain may be fucosylated by enhanced activity of alpha 1 > 3 fucosyltransferase leading to the accumulation of Le^x hapten (or X-hapten) glycolipid in some tumors; and (3) the type 1 chain may be fucosylated by enhanced alpha 1 > 4 fucosyltransferase as well as alpha 1 > 2 fucosyltransferase to accumulate both Lea and Leb haptens, regardless of the blood group Lewis status of the host. More recent studies designed to elucidate the nature of human TAA that reacts with various antihuman tumor MAB (e.g., MAB against colonic, gastric, and pulmonary carcinomas and myelogenous leukemic cells) have revealed the antigenic determinant of these antitumor antibodies to be Le^x (or X) hapten.[73] However, Le^x is widely expressed in a variety of normal tissues (e.g., gastrointestinal and renal tubular epithelium, granulocytes, etc.) and therefore antitumor antibodies with Le^x specificity will have little usefulness for drug-targeting. Several more of the "antihuman tumor" MAB have been found to detect blood group determinants, e.g., an antipancreatic tumor antibody showing blood group B glycolipid specificity, an antihuman colon carcinoma antibody showing anti-A activity, antibodies against epidermoid carcinoma and antihuman lymphoid cell lines showing anti-H substance activity, and several MAB against human colonic, gastric, and pancreatic adenocarcinomas showing activity against antigens of the human Lewis blood group system.[68,74] Tumor-associated Lewis antigens may be useful markers for drug-targeting in cancer patients who are nonsecretors of Lewis substance since Lewis antigens are expressed in epithelial tumors, irrespective of the original secretor (Se) phenotype. Systematic analyses of human cancer cells with MAB have revealed novel dimeric and trimeric structures of X and its sialylated forms in human colonic cancer cells. MAB against these new antigens did not react with monofucosyl X or short-chain sialosyl X[68] and thus may prove to be fairly cancer specific.

Accumulated type 2 chain precursors have been detected as glycoproteins with Ii activity in two cases of gastric cancer, whereas accumulation of unsubstituted type 1 chain led to appearance of antigen moieties unrelated to Ii and the production of antitumor autoantibody in a patient with bronchogenic carcinoma.[68] There is also evidence that unbranched type 2 chain can accumulate in human leukemic cells, probably due to the blockade of synthesis of blood group determinants.[75]

Aberrant (or incompatible) blood group substances have been noted in various adenocarcinomas, e.g., the appearance of A-like antigen in 10 to 15% of gastrointestinal carcinomas of O and B group individuals.[76,77] This provides a possible model for the use of a patient's own anti-A antibody for drug-targeting. The chemical basis of the emergence of A-like antigen in their tumor cells is not very clear. It is possible that the aberrantly expressed A antigen in at least some patients is Forssman antigen since the latter cross-reacts with blood group A. Forssman antigens have been detected in human lung, gastric, and breast cancer cell lines.[68] Other rare examples of the aberrant ap-

pearance of blood group antigens in tumors include the detection of P-like or P_1-like antigens in tumors of a patient with pp genotype[68] and the appearance of a MAB-defined Burkitt's lymphoma-associated antigen, i.e., a globotriaosylceramide.[78] It appears that Burkitt's lymphoma lymphocytes express the pK antigen irrespective of the P blood group status of the patient. This antigen is present in a large variety of normal cells but is not expressed on the surface.

D. Tumor Markers Used for Targeting

Some of the common human tumor markers and associated tumors are described below. Tumor markers have been classified into three somewhat overlapping groups, i.e., (1) products of activation of fetal genes, (2) products of ectopic activation in tumors of genes involved in the synthesis of pregnancy-associated proteins, and (3) adult or fetal tissue-specific enzymes or isozymes that may be synthesized in tumor cells. In addition to their use for tumor identification and detection, antibodies to many have been used for drug or radionuclide targeting and the following section outlines markers that have been used or have potential for targeting.

1. Products of Expression of Fetal Genes

a. Carcinoembryonic Antigen (CEA)

CEA is synthesized by a large variety of epithelial tumors, especially cancers of the colon and rectum, breast, pancreas, stomach and lung, and by a proportion of cancers of the prostate, bladder, ovary, uterus, and thyroid. It has also been found in neuroblastomas and osteosarcomas. The term CEA designates a heterogeneous group of rather complex glycoproteins; the protein-to-carbohydrate ratio in CEA from different tumors has been found to vary from 1:5 to 1:1. Polyclonal antisera against CEA cross-react with a number of normal tissue antigens, including several biliary glycoproteins, an antigen in normal feces, colon, and normal gastric juice.[79,80] Cells in a number of benign lesions produce CEA, e.g., hyperplastic gastrointestinal epithelium in ulcerative colitis and Crohn's disease, intestinal polyps, benign prostatic hypertrophy, bronchitis, etc.[81,82] With the availability of MAB, a paramount question is whether there are epitopes in the CEA molecule that are specific for cancer. Accolla et al.[83] reported the production of two anti-CEA MAB that had very limited cross-reactivity with normal colon antigens. Most of the 20 anti-CEA MAB studied by Grunert et al.[84] could not distinguish epitopes expressed in CEA from epitopes expressed in other cross-reacting molecules. On the other hand, by studying the pattern of reactivity of eight anti-CEA MAB, Hedin et al.[85] were able to conclude that there are at least six different epitopes in the peptide moiety of CEA. Two of the MAB cross-reacted with normal colon antigens but none with biliary glycoproteins. The results of Primus et al.[86] on the reactivity of their anti-CEA MAB are consistent with the pattern of epitopes suggested by Hedin et al.[85] In the context of their carrier role, three points should be made about anti-CEA MAB: (1) the pattern of reactivity of anti-CEA MAB obtained after immunization with tissue preparations other than colorectal cancers suggests that CEA preparations from histologically different tumors contain epitopes specific for that type of tumor;[87] (2) at least some of the anti-CEA MAB show no additive binding when the various antibodies are mixed, indicating that one or more can react with a spectrum of antigenic sites;[88] and (3) some of the anti-CEA MAB have very low affinity and low binding capacity for CEA compared to polyclonal antibodies of goat or rabbit origin, even when the former shows a degree of specificity for CEA.[88,89] In a study using three MAB directed against three different epitopes of CEA and xenografts of three different CEA-producing human tumors, factors that determined tumor-specific localization were found to include the concentration of CEA in the tumor and the target epitope of the MAB.[85,90] An ^{131}I-labeled polyclonal anti-CEA antibody inhibited the growth of

a CEA-producing human tumor xenografted in hamsters.[91] MAB directed against certain epitopes of CEA have been found to cross-react with antigenic moieties on circulating cells. These MAB did not localize in the target tumor and caused systemic toxicity.[92]

In the context of anti-CEA antibody-mediated targeting of drugs, expression of CEA by hyperplastic or benign epithelial lesions does not constitute a problem. On the other hand, high levels of circulating CEA and cross-reactivity of anti-CEA antibodies with normal cells limit the use of this tumor marker for drug-targeting. Production by tumors usually elevates the level of the antigen in circulation. Although it has been claimed that circulating CEA does not prevent the binding of anti-CEA antibody to CEA-producing tumors in vivo,[93] the possibility remains that elevated levels of circulating antigen will interfere with the binding of anti-CEA antibody to the target tumor and may give rise to circulating immune complexes. Radiolabeled anti-CEA antibodies have been used for the imaging of various human carcinomas[93] and as carriers of cytotoxic agents (see Ghose et al.[14]).

b. Alpha Fetoprotein (AFP)

AFP is produced by hepatocellular cancers, gonadal and extragonadal germ cell tumors (especially those that contain yolk sac elements), and cancers of the stomach, pancreas, and lungs. AFP from various types of cancer is much less heterogeneous than CEA and is antigenically identical to fetal AFP. It is synthesized by proliferating liver cells, especially during hepatocellular regeneration. AFP production by tumors usually leads to the elevation of serum levels. Anti-AFP antibody has been found to inhibit the growth of AFP-producing tumors in vitro, of rodent tumors in vivo, and of AFP-producing human tumors in nude mice.[82,94] Anti-AFP antibodies have been used for radioimmunodetection of cancer[93,94] and as carriers of cytotoxic agents.[14] A number of anti-AFP MAB have been produced (listed recently by Sell[94]). Four epitopes have been identified on AFP with the use of panels of these MAB.[95] Most anti-AFP MAB appear to have less affinity than their conventional polyclonal counterparts.

2. Pregnancy Proteins

These proteins (or antigenically closely related substances) may be produced by trophoplastic tumors or ectopically by various nontrophoplastic tumors.

a. Human Chorionic Gonadotropin (hCG)

Production of hCG occurs in trophoblast and germ cell-derived tumors and also ectopically in a proportion of other tumors, e.g., ovarian, breast, gastrointestinal, liver and lung cancers, melanomas, islet cell tumors, etc. In addition to trophoblastic tissue, small amounts of hCG are present in testis, pituitary, and gastrointestinal tissue.[96,97] The alpha chain of hCG is shared by LH and other glycopeptide hormones. Although a portion of the beta chain (i.e., N-terminal amino acids 116 to 145) is responsible for its antigenic identity, the beta subunit also cross-reacts with other glycopeptide hormones, especially LH. Antisera raised against the unique carboxy terminal peptide of the beta subunit have essentially no cross-reactivity with hLH.[98] A large number of MAB have been produced against hCG and its subunits.[99] These show a wide range of affinity and cross-reactivity. There appear to be four major epitopes each in the alpha and beta subunits. In regard to the carrier role of anti-hCG MAB, it is interesting that certain pairs of MAB display synergism in binding. The mechanism involves the formation of a circular complex consisting of two antigen and two antibody molecules.[99] Synthesis of hCG by tumors also is usually accompanied by an increase in the level of hCG in circulation. Furthermore, there is usually an increase in the circulating level of LH in gonadectomized patients. Elevated serum levels of hCG or LH, that cross-react

with anti-hCG antibody, might interfere with the tumor localization of the antibody. Some tumors produce only isolated free subunits of hCG that might not react with a given antibody.[97,100] Antibodies to beta-hCG have been used for tumor imaging.[93]

b. Placental Lactogen (hPL)

Apart from trophoplastic tissue, hPL is produced in small amounts by normal testis. It is produced by both male and female choriocarcinomas and a large proportion of breast cancers.[101] Compared to hCG, the serum level of hPL is usually lower in most patients. With residual tumor, hPL becomes undetectable in serum, whereas hCG can still be demonstrated.[102] Thus, for drug-targeting, hPL may be a more suitable target, provided that adequate amounts are produced by target tumor cells.

c. Pregnancy-Specific Glycoproteins

These include SPI alpha and SPI beta (synonyms: trophoblast-specific beta globulin [T BGi]; pregnancy associated plasma protein C [PAPP-C]; and pregnancy-specific beta, glycoprotein). In addition to trophoblastic tumors, immunoreactive SPI has been found in 60% of breast cancers and 50% of malignant gastrointestinal epithelial tumors.[101] They have also been found in the breast duct epithelium in a proportion of patients with benign breast disease.[97] In both trophoblastic and nontrophoblastic tumors, the serum levels of SPI have been found to be low compared to that of hCG. After chemotherapy, circulating SPI disappears much earlier than hCG.[103] Thus, there may be less interference with the carrier antibody by circulating SPI.

d. Placental Protein 5 (PP5)

PP5 is present in small amounts in the cytoplasm of syncitial trophoblasts and in the cytoplasm of breast carcinoma cells.[97] Adequate studies have not been carried out to establish its presence in other tumors or in the serum of cancer patients.

3. Tissue-Specific Enzymes

a. Acid Phosphatase (E.C.3.1.3.2)

Prostatic carcinomas contain different proportions of normal isozymes of acid phosphatase.[104] The serum level of PAP is raised both in prostatic carcinoma and benign prostatic hyperplasia. Antibodies to PAP cross-react with acid phosphatase in other tissues (i.e., liver, spleen, bone, kidney platelets, leukocytes, and erythrocytes).[104] Antibodies to PAP have been successfully used for imaging human prostatic carcinoma.[17,93]

b. Alpha Lactalbumin

Alpha lactalbumin is exclusively present in mammary tissue and its level goes up in mature breast tissue as a result of prolactin stimulation. It is the B protein of lactose synthase (E.C.2.4.1.22). The A protein is present in a higher proportion of breast cancers than the B protein but occurs in various other tissues. The B protein has also been found in the serum of breast cancer patients.[105]

c. Alkaline Phosphatase (E.C.3.1.3.1)

All alkaline phosphatase variants associated with cancer have counterparts related to early fetal development, i.e., these are fetal isozymes recurring in cancers. Various human cancers such as hepatomas produce the Regan or Kasahara isozyme.[105] It closely resembles the B variant found in human placenta. This isoenzyme is ectopically expressed on the surface of tumor cells in 12% of different types of carcinoma but in over 50% of ovarian carcinoma and seminoma.[106] There is elevation in the serum level in patients who have these isozyme-producing tumors.[105] A MAB against this isozyme

has been successfully used to target ricin A chain against a human cervical carcinoma line in vitro.[107]

d. Alcohol Dehydrogenase Isozymes (E.C.1.1.1.1)

In some hepatomas there is reexpression of the early fetal form of isozyme aa.

e. Creatin Kinase (CPK) (E.C.2.7.3.2)

The isozymes of CPK are fairly tissue specific. MM and BB are the only isozymes in muscle and brain, respectively. BB predominates in fetal tissues and adult gastrointestinal tract, uterus, and prostate. Tumors continue to produce the isozyme typical of the tissue of origin, although there may be reexpression of the embryonic form BB.[105] In patients, there is usually elevation of the serum level of the isozyme produced by the tumor.

f. Salivary Amylase (E.C.3.2.1.1)

Some lung and ovarian cancers produce salivary amylase, whereas only small amounts are produced by normal lung tissue. Amylase produced by tumors may differ slightly from salivary amylase in its carbohydrate moiety.[105]

g. Pancreatic Amylase

This amylase is produced by cancer of the pancreas, leading to elevation of serum amylase in patients.

E. Lymphoma/Leukemia Markers

The use of various polyclonal and MAB to study the cell surface antigen(s) in lymphomas/leukemias has provided insight into the ontogeny, differentiation, and function of various subpopulations of cells in the lymphoid/hematopoietic systems.[108] Precise schemes of T-cell differentiation and function have been delineated with MAB reagents,[109,110] but markers of B-lymphocyte differentiation have not been as well defined. Antibodies to HLA-DR[111] and to surface Ig were the principal reagents, but recently a number of MAB to other B-cell differentiation antigens have become available. Several excellent reviews have appeared on the surface markers of both normal and malignant T, B and other lymphoid and hemopoietic cell populations, and the various MAB that help in their identification.[112-117] Characterization of the differentiation antigens on T- and B-lymphocytes shows that many of the leukemia-specific or leukemia-associated antigens identified with polyclonal antibodies are in reality differentiation antigens.[116,117] For example, CALLA, originally defined with rabbit antisera after immunization with SIg$^+$, ER-ALL cells, and more recently with MAB J5, is present in about 70% of patients with all types of ALL and also in lymphoid B-cell precursors in normal bone marrow.[117] Several of these "antileukemia" MAB have been used in the serotherapy of leukemia in spite of their lack of specificity.[118] Most MAB against nonlymphoblastic leukemias only detect lineage-restricted normal hematopoietic differentiation antigens, and there is no evidence that these MAB can distinguish leukemic stem cells from normal pluripotent hematopoietic stem cells.[119]

Several MAB appear to demonstrate a fair degree of tumor specificity. For example, Naito et al.[120] have described two (MAB 3-3 and 3-40) that reacted with T-ALL cells but not with normal hematopoietic cells, and Negoro and Seon[121] obtained several that reacted with both acute and chronic T-leukemia cells. Berger et al.[122] reported MAB BE-1 and BE-2 that reacted with two different antigens on cutaneous T-cell lymphoma but not on normal T-cells. Virus-associated antigens of HTLV1 appear to be a potential tumor-specific target for MAB. Several MAB are available for HTLV internal core proteins including p19 and p24 HTLV proteins that appear to be membrane associated,

i.e., either on the inner surface of the T-cell membrane or inside HTLV on the infected T-cell surface (see Harden and Haynes).[117] Also, MAB that are directed against proteins outside the virus core are becoming available, e.g., MAB HT462 that is specific for an antigen expressed by HTLV and by HTLV-infected cells.[123] As already stated, the idiotype of the Ig molecule on B-cells offers a very specific target.[29]

F. TAA in Solid Tumors

In this section, without being comprehensive, information on human TAA that have been fairly well characterized (and therefore can be used for the production of well-defined anti-TAA antibodies) and some of the MAB against human TAA that appear to be potentially useful for drug-targeting will be briefly summarized. Two more comprehensive reviews on MAB against human TAA have been published recently.[124,125] Evidence for the specificity of most MAB-defined human TAA is inadequate. Many are oncofetal or differentiation antigen(s), while others merely reflect quantitative changes rather than *de novo* synthesis of antigenic moieties. Use of sensitive assay systems and careful examination of various normal cell populations at different stages of differentiation and different phases of the cell cycle have raised the question of whether human tumor antigens with unique specificity really exist.[126]

It has been proposed[127] that MAB-defined TAA can be classified into three categories: class 1 antigens that are restricted to autologous tumor cells (i.e., individual tumor specific); class 2 antigens that are common to histologically similar tumors from several patients but absent from other tumors and normal tissues; and class 3 antigens that are common to tumor cells and various components of normal tissue. Antibodies against class 2 antigens appear to be the most useful for targeting. In regard to class 1 antigens, every tumor has to be carefully analyzed serologically for the detection of the unique TAA. This is likely to be a time-consuming process jeopardizing therapeutic effectiveness. Most anti-TAA antibodies (irrespective of whether they are polyclonal or monoclonal in origin) appear, on close scrutiny, to be directed against class 3 antigens. However, this is not a serious limitation of their usefulness as carriers. A substantial quantitative difference in the distribution of the target antigen between the tumor and surrounding normal tissue may provide an adequate gradient for the localization of the carrier antibody, provided that the presence of cross-reacting antigen in the circulation or any other accessible tissue does not divert the antibody away from the tumor. Furthermore, localization of cytotoxic-agent-antibody conjugates in TAA-bearing non-vital normal tissue(s) may not be an insurmountable limiting factor.

1. Malignant Melanoma

Interest in the immunotherapy of human melanoma has led to extensive investigations on human MAA using both polyclonal antibodies[128,129] and MAB. More than 30 different MAB against MAA have been produced.[129-135] Most of the MAB-defined MAA are proteins but some are glycoproteins, proteoglycans,[129] or glycolipids.[134] The p97 MAA appears to be closely related to transferrin.[130,136] The list of MAA includes DR and other alloantigens, nerve growth factor receptors, and antigens shared by melanomas, nevus cells, tumors of neural crest origin, and various other tumor and normal fetal or adult cells.[134,137] However, with the probable exception of MAB D 1.1 (directed against aberrantly O-acetylated GD_3 diasialoganglioside),[138] no MAA with exclusive melanoma specificity have been identified using MAB.[134] Nevertheless, a number of anti-MAA have been demonstrated to selectively localize in human melanoma in nude mice and patients.[16,139-143] There was also inhibition of growth of human M21 melanoma cells in nude mice after treatment with MTX conjugated to MAB 225.28S.[144] MAB of human origin have also been produced against ganglioside GD2,

a membrane ganglioside present on human melanomas, retinoblastomas, and neuroblastomas.[145]

2. Cancers of the Gastrointestinal Tract

Radiolabeled anti-CEA antibodies have been widely used for tumor detection in vivo[146,147] and as carriers of cytotoxic agents.[14] Other potentially useful markers of carcinomas of the gastrointestinal tract include the following.

a. Fetal Sulfoglycoprotein Antigen (FSA)

FSA is found in fetal gastrointestinal tract mucosa and gastric cancer. It is not usually found in normal adult gastric tissue but may be present in the involved gastric mucosa in diseases such as atrophic gastritis, peptic ulcer, gastritis, and polyps. Investigations suggest that FSA is a component of CEA of colonic origin.[148]

b. Gastric Carcinoma Sulfoglycoprotein Antigen (SGA)

This antigen is present in fetal intestine, adult normal colonic mucosa, and most commonly in well-differentiated mucinous adenocarcinomas but not in undifferentiated gastric adenocarcinomas.[149]

c. Glycolipids of Gastric Cancer

In general, human gastric cancer tissue contains more neutral glycolipids than does normal mucosa. Glycolipids of the lactose series, including fucolipids, are markedly increased in cancer. Blood group-A-like substances have been found in the tumor tissue of two O group patients but not in surrounding uninvolved mucosa.[150]

d. Fetal Gut Antigen (FGA)

Smith and O'Neill[151] obtained an antibody against a FGA which reacted with 87% of fetal gut extracts, 30% of gastrointestinal carcinomas, and 8% of normal gut tissues but not with the serum of patients with gastrointestinal cancer. Therefore anti-FGA antibody, in appropriate patients, could be a better carrier of cytotoxic agents than anti-CEA antibody. Unfortunately, FGA and several other similar antigens were mainly defined by polyclonal antibodies and have not been adequately characterized biochemically. Furthermore, how different these oncofetal antigens are from CEA is open to question.

e. Zinc Glycinate Marker (ZGM)

Pusztaszeri et al.[152] identified a 2-kdalton antigen with an alpha-2 electrophoretic mobility in well-differentiated adenocarcinomas of the colon. This antigen has been found also in gastric, breast, prostatic, and lung cancers. However, the antigen was found in normal colonic tissue in patients with colonic cancer and nonmalignant gastric, pyloric, and small bowel mucosa.

f. Colon-Specific Antigen P (CSAp) and Colon Mucoprotein Antigen (CMA)

Radiolabeled antibody to CSAp[153] could localize xenografts of a human colon carcinoma in hamsters better than anti-CEA antibody.[154] Goldenberg's group has also reported alterations in the antigenicity of a normal colon-specific mucoprotein antigen (CMA) in colonic adenocarcinomas.[148]

g. MAB-Defined Antigens

Steplewski and Koprowski[155] have listed the various human colorectal carcinoma antigens defined by MAB. These are either glycoproteins (a few being identical with CEA), neutral glycolipids, monosialogangliosides, or members of the Lewis blood

group antigens. None are strictly colorectal carcinoma specific and a few react with erythrocytes or granulocytes. MAB 19.9 is directed against a ganglioside with a sialylated Lewis A carbohydrate.[137] This TAA is expressed by adenocarcinomas of the gastrointestinal tract, including pancreas, and by intestinal polyps. It is present only in a single layer of epithelial cells lining bronchi, pancreatic ducts, etc. in the saliva of Le^{a+b-} and Le^{a-b+} individuals and in meconium. Although this TAA is present in the serum of patients with gastrointestinal cancer, radiolabeled MAB 19.9 has detected gastrointestinal cancers.[156]

3. Liver Tumors

AFP has been the most used marker of human hepatomas. Antibodies to AFP have been used for radioimmunodetection of AFP-producing tumors and as carriers of cytotoxic agents in experimental tumor models (see Ghose et al.[14]). Immunohistological methods have demonstrated hepatitis B virus antigen in the liver tissue of most hepatoma patients. However, it is most abundant in cells that do not appear to have undergone malignant transformation.[157]

4. Pancreatic Carcinoma

Due to the difficulties in early diagnosis, there has been an extensive search for clinically useful markers of pancreatic cancer. CEA has been fairly widely used for this purpose. However, the presence of CEA or antigenically similar substances in other cancers and in normal and certain pathological tissues has severely limited its usefulness as a specific marker of pancreatic cancer.[158] Using homogenates of whole fetal pancreas, Banwo et al.[159] claimed to have obtained a polyclonal serum that could detect a pancreas-specific oncofetal antigen, i.e., pancreatic oncofetal antigen (POA). Though initially POA was found to be elevated only in the serum of patients with cancer of the pancreas, more extensive studies revealed elevated serum levels in other cancers and even in normal individuals with liver disorders.[160] In many of the earlier investigations, POA was not biochemically characterized and it was not even certain whether the different batches of polyclonal sera used in these studies were detecting the same antigen. Recently, two pancreas-specific antigens, i.e., one designated PaA (pancreas-specific antigen, a single chain protein of molecular mass 44 kdaltons)[161] and another, a pancreatic duct mucin-specific antigen,[162] have been defined by polyclonal sera. Both are expressed by at least a proportion of human pancreatic adenocarcinomas. Several TAA of human pancreatic carcinomas have also been identified by polyclonal antibodies[160,163] and MAB.[53,164-167] Most pancreatic TAA lack specificity, e.g., the heavily glycosylated mucin-like antigen detected by DU-PAN-2 is present on normal pancreatic ductal epithelium.[164] MAB AR2-20 and ARI-28 (directed, respectively, against 190- and 10-kdalton moieties)[167] and MAB C-P83 (directed against a 100-kdalton moiety)[168] appear to be fairly tumor specific after somewhat limited screening. However, the serum level of both organ- and tumor-associated pancreatic antigens is elevated in most patients with cancer of the pancreas.[161,169] For a more detailed description of MAB- and polyclonal antibody-defined tissue and tumor-associated antigens of the pancreas, see Sell and Reisfeld.[124]

5. Breast Tumors

As with other carcinomas, CEA has been the most used marker of mammary carcinomas. In a search for more reliable markers, Imam[170] has evaluated the potential clinical usefulness of various breast cancer-associated markers. These include: (1) ectopic products of breast tissue, e.g., a lactalbumin, casein, lactoferrin, and the milk globule membrane; (2) oncofetal antigens, e.g., CEA, beta oncofetal antigen (OFA); (3) hormones, e.g., hCG, calcitonin, and steroid receptors;[171] (4) enzymes, e.g., Regan

isotype of alkaline phosphatase, sialyl transferase, etc.; (5) pregnancy-associated glycoproteins;[172] (6) ferritin; (7) T-antigen; (8) nucleosides; (9) polyamines; (10) viral proteins. None appear to have adequate specificity or sensitivity for diagnostic or therapeutic purposes. Earlier investigations using autologous sera from patients demonstrated that there were antibodies that reacted with antigen(s) in breast tissue in a large proportion of breast cancer patients. However, the nature of these antigens and their tumor specificity were not adequately established. Later attempts at isolation yielded TAA, most of which showed substantial cross-reactivity with CEA.[148]

Increasing numbers of reports are appearing on MAB that react with human mammary carcinomas. They have been classified into four groups on the basis of their method of production and the immunogen used. One procedure uses lymphocytes from lymph nodes draining breast cancer, i.e., B-cells sensitized against TAA in vivo. Several of these MAB could discriminate between normal and malignant mammary epithelium.[172] However, all showed reactivity with epithelial cells lining renal tubules and sebaceous glands and therefore their therapeutic potential appears to be limited. Other procedures use splenic cells from mice immunized with human breast cancer cell lines, milk fat globule membrane, or crude or membrane-enriched fractions of primary or metastatic breast cancer. Schlom et al.[173] have listed the available MAB against human breast cancer and the nature of the antigen with which they react. Although it has been claimed that some react specifically with mammary cancer cells[174,175] or with both normal and neoplastic mammary epithelium,[176] none are specific for breast cancer and all appear to be antibodies against class 3 TAA. Furthermore, every MAB behaves differently with respect to "percentage of reactive mammary tumors, percentage of reactive cells within tumors, cellular location of the TAA, and extent of reactivity with non-mammary tumors and normal tissues".[173] In spite of these limitations, specific localization of radioactivity in breast cancer has been demonstrated after administration of radiolabeled polyclonal antibodies (or their reactive fragments) against CEA[177] or hCG[178] and radiolabeled MAB against mammary cancer (see Schlom et al.[173]).

6. Prostate and Prostatic Carcinoma

A number of human prostate-specific antigens including PAP have been isolated and several have been biochemically characterized.[104,179] As carriers of cytotoxic agents in the treatment of primary and metastatic carcinoma of the prostate, antibodies to prostate-associated antigens are likely to be useful provided that these normal tissue markers are adequately expressed by the tumor tissue. The tissue-specific antigens, prostatic antigen (PA) and PAP are produced by the majority of primary and metastatic prostatic carcinomas, although PAP production may be reduced in tumor tissue, especially if the tumor is undifferentiated.[104,180,181] The serum levels of these markers are elevated in patients with prostatic cancer. Several MAB have been obtained against different prostatic TAA[180,182,183] and normal PA,[184] including PAP.[104,185] MAB against different epitopes of PAP[185] and a prostatic TAA[186] have also been obtained. MAB D83.21 against prostate carcinoma also reacts with human bladder cell lines,[182] and the antigen recognized is a membrane glycoprotein with 60- and 28-kdalton subunits cross-linked through disulfide bonds.[187] Both polyclonal antibodies and MAB against PAP have been demonstrated to localize in xenografts of human prostatic carcinoma.[17,188] It has been claimed that anti-PAP MAB-linked fluorouracil deoxyriboside inhibited the growth of a prostatic carcinoma xenograft in nude mice.[188] Although the serum levels of PAP, prostate-specific, and prostatic TAA are usually elevated in patients with prostatic cancer, the serum levels of all the markers are not raised in all patients.[185,189] For agents that need endocytosis, it is pertinent that the cell surface-located prostatic TAA moiety P54 is not shed but internalized after binding to MAB alpha

Pro3 or alpha pro5. These two MAB bind to P54 via different epitopes and show a synergistic effect on endocytosis.[186]

7. Carcinoma of the Ovary

Adenocarcinoma of the ovary is the most lethal gynecological cancer and its curability falls sharply with each advance in the stage of the disease. Both AFP and hCG have been widely used as markers of ovarian germ cell tumors. AFP is associated with endodermal sinus tumors and embryonal carcinomas, while hCG is associated with choriocarcinomas and embryonal tumors but not with endodermal sinus tumors. CEA is not a useful marker for most of the epithelial tumors.[190] Before the advent of hybridoma methodology, autologous sera from patients with adenocarcinoma of the ovary or xenogenic immune sera were used to define putative ovarian carcinoma-associated antigens. Except for one,[191] they have been poorly characterized and a few proved to be identical with CEA.[148,190] Bhattacharya et al.[192] identified six different ovarian carcinoma-associated antigens in ovarian cytoadenocarcinoma. Other ovarian TAA include the glycoproteins OCA[193] and CA 125.[194] However, most of the antibodies cross-react with fetal or adult intestine[192] and/or other tumors.[195,196] MAB DU-PAN 2 raised against pancreatic carcinomas reacts with approximately 40% of epithelial ovarian tumors. The CA 19-9 antigen, originally detected in a colorectal carcinoma cell line, has a sialylated Lewis blood group A determinant on a mucin-like glycoprotein (Mr > 500 kdaltons) and CA 19-9 is found in approximately 40% of ovarian carcinoma tissues and in the serum of 20% of ovarian carcinoma patients.[190] The serum level of other ovarian TAA is usually elevated in these patients as well as in a proportion of patients with other nonovarian adenocarcinomas.[197] Radiolabeled antibodies against CEA and ovarian TAA have been used for imaging and treating ovarian cancer.[198-202]

8. Lung Cancer

Early attempts at the identification of TAA of lung cancer using immune xenoantisera led to the recognition of several antigens that were distinct from CEA, AFP, and ferritin, e.g., antigens X and Y[203], HLTAA, LTA I, and LTA II.[204] However, most were not adequately characterized or properly evaluated in regard to their specificity.[148,205] It is now realized that human lung cancers may be divided into two broad categories, i.e., small cell lung cancers (SCLC) and non-SCLC that include squamous, adenocarcinomas, and large cell cancers. SCLC and bronchial carcinoids are related to or arise from pulmonary endocrine cells having APUD properties (i.e., cells that have the ability for amine and precursor uptake and decarboxylation) and therefore it is not surprising that many eutopic and ectotopic hormone markers are produced by SCLC tissue. These include the APUD cell marker, L-dopa decarboxylase, neuron-specific enolase, the specific peptide product bombesin, enkephalins, and calcitonin. Other peptide products elaborated most often by SCLC (and occasionally by non-SCLC) include arginine, vasopressin, and neurophysial oxytocin. Single SCLC tumors have been reported to secrete up to ten hormones. SCLC also produce non-APUD markers such as CPK and its BB isoenzyme.[205,206] In contrast to SCLC, which are most frequently associated with APUD cell products, non-SCLC are associated with both APUD and non-APUD hormones. For example, although clinical syndromes due to excessive production of ACTH secretion have been limited to SCLC and bronchial carcinoids, all types of lung cancer produce proACTH and lipotropin, a large protein that is a precursor to both proACTH and ACTH. Furthermore, all types of lung cancer cells secrete calcitonin and hCG. Some hormones are synthesized more commonly in non-SCLC than in SCLC, e.g., human placental lactogen and growth hormone. There is a frequent association between the bizarre giant cell type of large cell carcinoma and hCG secretion.[205]

Immunochemically, the hormones produced by malignant cells may be heterogeneous, e.g., calcitonin,[207] or may consist of various free subunits, e.g., hCG. In the context of tumor markers, SCLC tend to produce ectopic polypeptide hormones such as ACTH and bombesin and are rich in neuron-specific enolase, L-dopa decarboxylase, and creatine phosphokinase BB.[206] MAB have been produced that react with both SCLC and non-SCLC,[208] with non-SCLC only,[206] with mainly squamous cell carcinomas,[209] or with human cancer cell lines.[210,211] The MAB 534 F8 against SCLC generated by Cuttitta et al.[208] recognizes lacto-*N*-fucopentose III. This oligosaccharide is expressed in murine embryos and embryonic carcinomas (SSEA-1 antigen), normal human bronchial epithelium and myeloid cells, and various proportions of other human cancers, e.g., NSLC, colon and breast cancers, and malignant cells of Hodgkin's disease.[124] Other TAA found in lung cancers of diverse histology are CEA-related antigens and an antigen that inhibits alpha-1-chymotrypsin.[212]

Recently, several MAB with more restricted reactivity have been obtained, e.g., MAB that react with lung tumors of a given histologic type such as squamous cell carcinoma, adenocarcinoma, and large cell carcinoma but not with SCLC, other human tumors, and normal tissues.[213] MOC-1 reacts with SCLC and normal Kulchitski cells that constitute a subset of normal endocrine and neural cells.[214] MAB, TFS-2, and SM-1 that, along with complement, specifically lyses SCLC cells but does not react with normal lymphoid or bone marrow cells have been successfully used to purge SCLC cells from human bone marrow.[215,216] However, there is considerable heterogeneity of antigen expression in lung tumors of even a given histologic type.[124] An IgM MAB, 600 D11, selectively localized in human SCLC xenografts in nude mice.[217] There is some evidence that MAB 211 (that has specificity for bombesin) and MAB 11G11 (that reacts with a subset of SCLC) inhibits target tumors in vitro and their xenografts in nude mice.[124]

9. Osteosarcoma

An MAB that reacts with several different human osteogenic sarcoma lines has been successfully used to image human osteogenic sarcoma xenografts in nude mice. Conjugates of this MAB with MTX, vindesine, adriamycin, or ricin A chain inhibited target cell lines.[218] However, it cross-reacts with other human tumor lines that are histologically different and also with some normal human tissues. Biochemically, it appears to be directed against an integral membrane protein of 72 kdaltons.[219] Hosoi et al.[220] have also produced several MAB that react with both human osteosarcoma and chondrosarcoma. Prior to this, appropriately absorbed polyclonal xenoactisera were shown to react with human osteosarcoma cells with varying degrees of specificity.[221]

10. Renal Cancer

An antibody against human renal cell carcinoma (RCC) was obtained by Ghose et al. after immunization of a RCC patient with a histologically similar allogenic tumor.[222] Ravitz et al.[223] also detected autoantibodies to RCC in the serum of RCC patients. By immunizing goats and rabbits with viable RCC cells and absorbing the immune sera with AB Rh+ human red cells and a panel of normal human tissues including kidney, Ghose et al. obtained antibody preparations that reacted with a large proportion of human RCC from different patients but did not react with any normal human tissues or with other histological types of human cancers.[224] Ghose et al. have also demonstrated that, after i.v. injection, these polyclonal anti-RCC antibodies selectively localized in tumor tissue.[225] Attempts have been made to produce RCC-specific MAB.[226-229] But, with rare exceptions,[226a] these antibodies cross-react with various other human tumors and normal tissues. The antigen detected by the MAB of Ueda et

al.[226] has been found to be the adenosine deaminase-binding protein.[230] Other TAA in RCC that can possibly be exploited for drug-targeting include renin[231] and steroid receptors.[232]

11. Urinary Bladder Cancer

As with renal cancer, investigations using either xenogenic antiserum[233] or autologous sera from bladder cancer patients[234] have suggested the existence of TAA in human bladder cancer. Fradet et al.[235] have used MAB derived from mice immunized with human bladder cancer cell lines or lysates of bladder papilloma to define 11 distinct antigenic systems. Two of these antigens, Om5 and J233, are not expressed by any cultured cells of normal origin or by normal and fetal tissues. Two other antigens, T101 and JP165, are subset markers for bladder cancer and are not detected in normal tissues. The remaining antigens are expressed by various other normal and neoplastic cells. All antigens detected by these MAB are heat labile and are not related to A, B, H, I, or Lewis blood group antigens.

12. Cervical Cancer

A number of TAA associated with squamous cell carcinoma of the cervix have been described using polyclonal xenoantibodies. Levi[236] obtained two precipitin lines after immunodiffusion of cervical carcinoma homogenates against a normal human tissue-absorbed rabbit immune serum obtained after immunization with homogenates of human cervical carcinoma. Levi's work stimulated a number of similar investigations with comparable results.[148,237] However, the biochemical nature of these antigens, the extent to which they are tumor or tissue specific, and the relationship among them remain controversial. Other TAA associated with cervical cancer include CEA and a beta-oncofetal antigen[238] which was demonstrated in 19 of 21 cervical carcinomas.

However, this beta-oncofetal antigen was also found in low concentrations in normal cervical tissue and in high concentrations in normal adult liver and kidney. Ghose et al. detected CEA in 16 of 16 squamous cell carcinoma of the cervix irrespective of whether the lesions were poorly or well differentiated.[239] The serum levels of CEA have been found to be elevated in a proportion of (but not all) cervical carcinoma patients.[237] The usefulness of beta hCG and AFP as markers of cervical cancer is controversial.[240] Herpes simplex virus 2 (HSV-2) antigens have been demonstrated in exfoliated cells of 100% of a group of patients with invasive and pre-invasive cervical carcinoma[241] and in 94% of another group with invasive cervical carcinomas.[242]

13. Neurogenic Tumors

a. Neuroblastomas

Neuroblastoma is a common childhood tumor that arises from primitive sympathetic neuroblasts[243] and presents diagnostic problems. Several MAB have been produced against human neuroblastomas. Examples include MAB P1-15 3/3 that reacts with neuroblastomas, glioblastomas, retinoblastomas, acute lymphoblastic leukemia cells (excluding leukemias of T-cell origin),[244] and MAB HSAN 1.2 that binds also to Wilm's tumors, Ewing's sarcoma, retinoblastoma cell lines, and fetal brain.[245] Similar cross-reactivity of other antineuroblastoma MAB with normal cells and tissues has been observed, e.g., with T-lymphocytes.[243] A MAB against the human Thy 1 antigen has also been found to react with neuroblastoma cells.[246] However, the Thy 1 antigen is expressed by various normal cell populations and tumors such as gliomas, myogenic sarcomas, teratomas, Wilm's tumors, and T-cell leukemias. Specificity analysis of MAB produced against tumors originating in neural crest-derived tissues has demonstrated extensive cross-reactivity among neuroblastomas, gliomas, melanomas,[247] and hematopoietic cell lines.[248]

Wilkstrand and Bigner[249] have recently listed MAB-defined human neuroectodermal tumor-associated antigens (HNTA). These include: (1) tissue-specific markers, many of which have been biochemically characterized (e.g., glial fibrillary acidic protein, S100, gangliosides, etc.); (2) shared nervous system-lymphoid antigens (e.g., Thy, HLA-DR, CALLA, etc.); (3) shared neuroectodermal and/or oncofetal antigens; and (4) putatively tumor-restricted antigens. The presence of markers under (1), (2), and (3) in one or more vital normal tissues (e.g., adult kidneys, brain, gastrointestinal tract, spleen, thymus, leukocytes, etc.) renders them unsuitable for tumor-targeting. Several MAB raised against melanomas have revealed specificity for gangliosides that are also expressed in gliomas and/or neuroblastomas and retinoblastomas, e.g., GD_2 (initially designated as OFA 1-2), GD_3, and GQ. Several anti-GD_2 MAB have also been produced after immunization of mice with human neuroblastoma cells.[250] In regard to normal tissues, GD_2 is mainly expressed by fetal brain. Although GD_3 is expressed by various normal tissues, e.g., normal brain, retina, and kidneys, MAB against melanoma-derived GD_3 binds poorly to normal tissues due either to a predominant intracellular location of the antigen or to minor chemical differences between melanoma-derived and normal brain-derived GD_3.[251] MAB P1 15 3/3[252] against a 30-kdalton glycoprotein is expressed in fetal brain and in glioma neuroblastoma and retinoblastoma cells as well as in B and null cell ALL and B-cell CLL. Antibodies against GD_2 and GD_3 and MAB P115 3/3 may have some potential for tumor-targeting. The specificity of those MAB that detect putatively restricted HNTA is yet to be established convincingly.

Most of the MAB against neuroblastomas bind to cell surface glycoproteins, many of which are probably differentiation antigens.[246] Recently, Cheung et al.[253] have produced a MAB against a cell surface glycolipid of somewhat restricted distribution, i.e., while the antigen is present on osteosarcoma and leukemic cells, it is absent from most Ewing's and Wilm's tumors. The MAB against this antigen reacted with more than 98% of cells in all surgically excised neuroblastoma specimens and were cytotoxic to 100% of neuroblastoma cells in the presence of complement. If the specificity of this antineuroblastoma MAB is confirmed, it appears to be very suitable for targeting chemotherapeutic agents in vivo. Some antineuroblastoma antibodies have been successfully used in treating autologous marrow,[254] localization of neuroblastomas in vivo,[255] and in treating neuroblastoma patients with chlorambucil- or daunorubicin-antibody conjugates.[256]

b. Glial Tumors

Appropriately absorbed xenogenic polyclonal antisera have detected common glioma antigens[249,257] and common astrocytoma antigens.[258,259] A rabbit antiglioma serum has detected a fetal brain antigen strongly expressed on gliomas and neurinomas.[260] A number of studies have demonstrated that sera from patients with brain tumors had a higher incidence of reactivity to tumor cells than did sera from normal donors.[261] MAB have been produced against human glioma-associated antigens by human-human,[262] human-mouse,[263] and mouse-mouse hybridomas. Although some of the MAB define antigens that appear to be shared and preferentially expressed by different histologic types of gliomas, none appear to be glioma or tumor specific. Vessels supplying intracranial tumors are not subject to the "blood-brain barrier".[249] Ghose et al. demonstrated that the $F(ab)_2$ moiety of a polyclonal antineuroblastoma antibody showed greater localization in mice than the parent IgG.[16,264] Furthermore, the delivery of MAB to brain could be increased fivefold by prior hyperosmolar perfusion of rats with 1.4 *M* mannitol or 1.6 *M* arabinose.[249] As already stated, many of the MAB produced against malignant melanomas, neuroblastomas, and hematopoietic cell lines also react with glial tumors.

14. Teratocarcinomas and Embryonal Carcinomas

Teratocarcinomas contain disorganized mixtures of adult, embryonic, and extraembryonic tissues that presumably arise by differentiation of pluripotent embryonal carcinoma cells.[265] Human embryonal carcinoma is believed to be of germ cell origin and multipotent in nature. Some embryonal carcinomas may be totally devoid of differentiation, whereas others may contain areas of choriocarcinomatous or yolk sac carcinoma differentiation, e.g., hCG and AFP, respectively. Several MAB have been raised against human teratocarcinomas, but none react exclusively with human embryonic carcinoma cells. For example, MAB 5.5.H., 8.7.D., and 13.7.A are directed against oncofetal antigens having extremely restricted in vivo tissue distribution.[266] The MAB described by Williams et al.[267] reacts with a 200-kdalton membrane glycoprotein on undifferentiated teratoma cells and cells lining human fetal intestine and bronchus. MAB TRA-1-60 and TRA-1-81 react with an antigen on undifferentiated embryonic carcinoma cells that is found also on other human tumor lines and several normal tissues.[268] A study using 3 MAB directed against high molecular weight glycoproteins of human teratocarcinomas and 165 human tumor lines and normal cells has confirmed the oncofetal nature of teratocarcinoma-associated antigens.[269] MAB against murine embryonic antigens such as SSEA-1, SSEA-3, and SSEA-4 react with moieties on the surface of human embryonic carcinoma cells or their differentiated derivatives even though there may be differences in the distribution of these antigens between the two species in regard to the differentiation status of tumor cell populations.[270] SSEA-1 is expressed by various normal and neoplastic human tissues.[271] Human embryonic carcinoma cells are SSEA-1 negative, although some components of germ cell tumors, e.g., yolk sac carcinoma and choriocarcinoma cells, express this antigen. The SSEA-1 epitope is a branched oligosaccharide that may occur as a part of a polylactosamide in the lacto-series of glycolipids. MAB against SSEA-3 react with human erythrocytes. The SSEA-3 epitope consists of the internal core of the oligosaccharide of globoside-7 (GL-7), whereas the SSEA-4 epitope consists of the terminal residues of this oligosaccharide. The globo-series of glycolipids contain the epitopes of the P blood group antigens.[272,273] Human teratocarcinomas produce very high levels of an alkaline phosphatase isozyme that is normally distributed in liver, bone, and kidney. After immunization of mice with a human teratocarcinoma line, two MAB against the liver/bone/kidney alkaline phosphatase isozyme have been obtained.[268] Ballou et al.[274] have successfully imaged xenografts of human teratocarcinoma in nude mice using a radioiodine-labeled IgM MAB against SSEA-1 and its $F(ab)_2$ moiety.[275]

III. PRODUCTION OF ANTI-TAA ANTIBODIES AND CRITERIA OF SUITABILITY FOR TARGETING

Details of the methods of production and purification of conventional polyclonal and monoclonal anti-TAA antibodies are beyond the scope of this review. The methods used by these authors for the production of polyclonal anti-TAA antibodies will be found in Ghose et al.[14] and other publications from the authors laboratory.[128,224,225,276] General methods for production of mouse hybridoma-derived MAB have been outlined[277,278] and methods for those against a given TAA are in the publications cited in Section II, e.g., colorectal carcinomas and melanomas,[156,247] antigens of human blood group systems,[74] lymphoma/leukemias,[120,121] ovarian carcinoma,[192] pancreatic carcinoma,[167] and mammary carcinoma.[173-175] The following comments are relevant to production of antibodies for targeting.

A. Rodent-Rodent Hybridoma Production

1. Selection of Species and Strains

Each species and strain of animal has a characteristic response pattern to a given antigen which must be taken into account. For example, some strains of rats do not respond to blood group A or B substances and are therefore suitable if one wishes to avoid the production of MAB against these antigens.[279] Whenever possible, more than one strain of mice should be used because the immune spleen cell donor and the immune response pattern of the species or strain of animal should be ascertained.

2. Antigen to be Used for Immunization

For the production of MAB against human TAA, the spleen cell donor can be immunized with: (1) tumor cell lines long maintained in culture; (2) freshly isolated tumor cells from patients; (3) membrane preparations or TAA-enriched fractions of cells from (1) and (2); and (4) serum free supernatants from the spent culture medium of tumor cells.[155,280] Tumor cells long maintained in culture tend to undergo antigenic modulation and lose certain antigens.[280] Their use yields MAB with low specificity, whereas use of freshly excised tumor tissue or TAA-enriched fractions derived therefrom yield MAB with more restricted specificity.[155,213] The use of pure TAA preparations yields highly specific MAB.[192,266] Methods of obtaining membrane preparations or TAA-enriched fractions from tumor cells have been outlined.[81,155,247,281] The use of formol-saline-fixed dehydrated tumor cells for obtaining a MAB that reacted with fixed tumor tissue has been reported.[267]

3. Immunization Protocol

For tumor localization in vivo, it is essential to have antibodies with high affinity. A single immunization of the spleen cell donor mouse is likely to yield low affinity antibodies so it may be advisable to use multiple injections, with or without adjuvants, for eliciting a secondary response.[155] The animals producing antibodies with the highest titer and affinity should be used for donating spleen cells for fusion. As most of the anti-TAA antibodies react in a limited way with one or more normal tissue components and no anti-TAA antibody reacts with a given histologic type of tumor, the reactivity with such tissues as renal glomeruli, bone marrow, etc. should be confirmed before use for targeting cytotoxic agents. Whenever possible, the tumor-specific localization of the carrier antibody should also be confirmed.

B. Human-Human and Human-Rodent MAB

The methodologies for the production of MAB of human origin are still in their infancy. Approaches that have been pursued[282] include the production of hybrids of lymphocytes from regional lymph nodes of cancer patients using rat or mouse myelomas,[172,283,284] human myelomas,[285] human B-cell lymphoma and lymphoblastoid lines,[282,286,287] or heteromyelomas.[282] An alternative approach for the production of human anti-TAA antibody-producing cell lines has involved the transformation of appropriately sensitized B-lymphocytes with Epstein-Barr virus.[282]

Human-rodent interspecies hybridomas are usually very unstable due to the selective loss of human chromosomes, and virus-transformed B-lymphocytes produce only small amounts of Ig and tend to cease antibody production after a variable period.[282,285] MAB of human origin are likely to be less immunogenic than xenogenic MAB for targeting anti-cancer agents in patients. However, the production of anti-idiotypic antibodies after repeated administrations of human MAB remains a possibility. Most human lymphocyte-derived MAB belong to the IgM class and have low affinity for TAA.[173]

C. Preparation of F(ab) and F(ab)$_2$ Fragments of Anti-TAA Antibodies

Several procedures for obtaining reactive fragments of Ig have been outlined elsewhere.[14,18] Lamoyi and Nisonoff[288] have demonstrated that mose hybridoma-derived MAB differ widely in their susceptibility to proteolytic digestion even within a given subclass. The products of digestion of MAB should be rigorously monitored using a two-dimensional gel electrophoresis along with appropriate marker proteins, and the reactivity of putative immunologically reactive fragments should be evaluated.[289,290]

D. Factors That Determine Tumor-Specific Localization of Antibodies

Factors that determine tumor-specific localization of systemically administered antibody preparations are outlined below. Many may be influenced by conjugation with agents (see below under methods of linkage).

1. Purity

The fraction of an antibody preparation that binds to available cell surface TAA is directly proportional to antibody purity.[289] Polyclonal sera are limited in their content of specific antibody even after affinity purification, which can yield preparations varying from 10%[291] to approximately 80% pure.[146] Furthermore, the extensive absorptions necessary for rendering polyclonal antibodies specific remove a substantial proportion of antibody molecules and leave behind contaminating antibodies that cross-react and bind to normal tissues. The antinormal tissue antibodies may contribute to systemic toxicity of conjugates.[292]

MAB, at least theoretically, consist of one clone of antibody molecules and therefore can overcome these difficulties. However, a source of inactive Ig molecules in MAB preparations is the secretion by the hybridoma of inappropriate hybrid Ig molecules that lack effective antibody-combining sites.[293] Also, in practice, when MAB are harvested from the ascites fluid of mice inoculated intraperitoneally with the antibody-producing clone, there is substantial contamination with mouse serum proteins that leak into ascites fluid. Isolation of MAB from ascites fluid presents in a limited way, the same problem as encountered during the purification of polyclonal antibodies. One method to overcome the problem is to grow the MAB-producing clone in vitro in serum-free medium[278] or in medium supplemented only with those components of fetal calf serum (FCS) that bind to protein A (if the MAB does not bind) and therefore can be removed by affinity purification with protein A. The problem of limited amounts of MAB that are usually obtainable from MAB-producing clones in culture (in contrast to harvesting MAB from ascites fluid) can now be overcome by methods for scaling up the production of MAB in vitro, e.g., either with the use of cytostat-growing clones inside hollow fibers or beads that are commercially available. Also, a commercial preparative HPLC purification system based on hydroxylapatite (Bio-Rad Laboratories, Richmond, Calif.) is applicable to ascites fluid.

Ghose et al. compared the tumor-specific localization of ^{131}I-labeled polyclonal anti-human melanoma IgG, anti-human melanoma MAB 225.28S, and normal mouse IgG in xenografts of human melanoma in nude mice. The tumor-to-blood ratio of radioactivity was highest with the MAB and lowest with the normal mouse IgG.[139] However several groups of investigators did not find any difference between monoclonal and polyclonal anti-CEA antibodies in regard to their localization in tumors and clearance from serum.[93] Stuhlmiller et al.[294] studied tumor localization of ^{131}I-labeled anti-human-melanoma antibodies of monkey, human (i.e., serum of patients immunized with allogeneic melanoma cells), and murine hybridoma origin in human melanoma xenografted nude mice. Compared with different control nonspecific IgG preparations, each specific antibody showed selective localization in tumor tissue. The ratio of tu-

mor-to-normal tissue localization was highest with the monkey antibody and lowest with the murine MAB.

2. Affinity for TAA

At a given antigen concentration, the affinity constant of an antibody determines the amount that can bind to target cells.[289] In turn, the amount of an antibody-linked drug (i.e., MTX) that is endocytosed is correlated with the amounts of carrier antibodies observed to bind at equilibrium at 0°C and the titer of the binding.[295] Antibodies that have low affinity, either intrinsically or as a result of the method of conjugation, are not likely to be effective for targeting. Even the fraction that binds is rapidly lost from the tumor.[296] Kennel et al.[289] have calculated that antibodies with binding constants of less than 108 M^{-1} are not likely to be useful for drug targeting or tumor imaging, assuming a value of 10^6 antigenic sites per cell. MAB are notorious for their low affinity. However, repeated immunization of splenic cell donor mice may yield hybridomas that produce high affinity antibodies.

3. Specificity

As already discussed, TAA that are exclusively tumor specific have yet to be found. However, it is the difference in the amount of the TAA (and the difference in their affinity for the antibody if any) in the target tumor and surrounding normal tissues that will be critical for drug-targeting. For example, oncofetal antigens that are not expressed or are expressed only in very limited amounts in normal adult tissues are likely to be adequate for drug-targeting. On the other hand, several anti-CEA MAB failed to localize in tumors and gave rise to systemic complications because of their cross-reactivity with CEA epitopes on normal cells.[92]

4. Concentration and Availabilty of TAA

The amount of antibody that will bind to tumor cells for a given MAB-TAA system will vary with the concentration of TAA sites.[289] The number of sites available on cells of experimental tumors has been calculated to be 2.5×10^4 to 7×10^6 per cell.[12,289] Results from our laboratory show that TAA sites on human melanoma cell lines fall within this range. According to Kennel et al.,[289] the TAA concentration in such a system is likely to be in the range of 10^6 to 10^9 *M*, but rarely above. The conditions that will determine the interaction between a fluid-phase drug carrying antibody and TAA in solid tumors are complex and include the following: (1) accessibility of TAA to conjugates in intravascular and extravascular fluids; (2) rate of diffusion of conjugates in the extravascular compartment; and (3) susceptibility of TAA to modulation and the rate of regeneration of TAA sites after endocytosis. TAA-antibody combinations in which the TAA modulates and becomes cryptic upon exposure to the antibody are obviously unsuitable for drug-targeting. Endocytosis of the carrier antibody is essential, if the cytotoxic agent is not surface active and its target molecules are intracellular. Comparison of the rate of endocytosis of three different MTX-linked antimelanoma antibodies has indicated differences in the rate of regeneration and recycling of TAA sites. The binding sites for MAB 225.28S regenerated faster and were more efficient for internalization of antibody-linked MTX than the binding sites of two polyclonal antimelanoma antibodies.[295] Some cell-surface TAA do not undergo endocytosis even after antibody-induced capping. For example, Ghose et al. observed capping and endocytosis of antibody-bound TAA on EL4 cells[297] but not with TAA on H6 hepatoma cells.[292]

5. Influence of Secreted or Shed Antigens(s) in the Millieu of Tumor Cells

Many TAA are either actively secreted (e.g., hormones, PAP, AFP, etc.) or shed

(e.g., MAA) in the extracellular fluid. Some TAA may also be released by dead or dying tumor cells. The concentration of such extracellular TAA is likely to be the highest in the immediate vicinity of tumor cells, especially in those areas where vascular flow is sluggish and lymphatic drainage is inadequate. The presence of extracellular antigen(s) in solid tumors is unlikely to be a serious impediment for imaging or therapy with antibody-linked radionuclides. In fact, the presence of large amounts of localized free antigens may increase the number of binding sites per unit volume of tumor tissue and thus lead to increased tumor localization of the agent. However, the presence of extracellular free antigen may interfere with the effectiveness of those conjugates of cytotoxic agents that need internalization for cytotoxic effect. On the other hand, dissociation of an active drug from the extracellular immune complexes may produce a local depot effect, i.e., a slow prolonged action of the agent at the tumor site. This will be unlikely to happen with immunotoxins constructed with only the A chain of the toxin molecule unless the A chain also contains the part of the B fragment (in a cryptic form) that facilitates internalization. Local extracellular accumulation of TAA-immunotoxin complexes may then result in tumor inhibition.

Apart from concentration, the affinity between free TAA and carrier antibodies is also an important factor in determining the effectiveness of antibody-linked agents. Interactions between antibodies and cell surface TAA can be regarded as a typical fluid phase mono- or bivalent reaction.[289,293] At least some of the membrane-bound TAA may behave atypically because of adjacent molecules (e.g., steric hindrance of glycolipid TAA by other membrane-located glycolipids,[298] membrane fluidity, and the relatively slow diffusion constants of membrane-bound antigens that may prevent the proper orientation of the epitope for binding with the antibody). Any advantage in affinity of extracellular TAA over cell surface-bound TAA will detrimentally affect the binding of carrier antibodies to tumor cell surface.

6. Interference by Host Antibodies and Immune Complexes

Although oncofetal antigens are usually not antigenic in the tumor host, other TAA (e.g., cell surface-associated neoglycoconjugates or blood group precsursors) may provoke a response in tumor hosts. Autologous anti-TAA antibodies may compete with drug-linked xenoantibodies for binding sites on the tumor cell surface. Relative concentrations of the two antibodies in the milieu of tumor cells and their affinity constants will be among the factors determining the amounts of the two antibodies that will bind to tumor cells. Autologous antibodies may react with circulating TAA to produce immune complexes which are likely to be in the zone of antigen excess at the tumor site. These complexes in antigen excess may also neutralize administered antibody conjugates. If the carrier antibody possesses a higher affinity for the TAA than the autologous antibody, then the carrier antibody is likely to displace the autologous antibody from complexes, causing further "neutralization" of the conjugate. Autologous antibody displaced from immune complexes will compete with the unbound antibody conjugates for TAA on the tumor cell surface. If the conjugated antibody happens to have a higher affinity for free TAA (or TAA in the immune complex) than for cell-bound TAA, there is likely to be substantial reduction in the binding of the conjugate to tumor cells. Furthermore, there will be additional loss of binding of the conjugate to tumor cells if the autologous antibody happens to have higher affinity for the cell-bound TAA than the carrier antibody.

7. Size of the Conjugated Antibodies

The use of F(ab) and $F(ab')_2$ fragments, instead of the entire Ig molecule, is likely to: (1) augment transcapillary passage and diffusion in extracellular space; (2) enable the carrier moiety to cross the blood brain barrier and thus make the carrier suitable

for the imaging or therapy of tumors in the central nervous system;[16,264] (3) reduce the antigenicity and susceptibility to phagocytosis; and (4) lead to faster clearance of the labeled moieties. Indeed, F(ab) and $F(ab')_2$ moieties of anti-TAA antibodies are cleared from the circulation at a much faster rate.[17] Rapid clearance of labeled carriers that are not tumor bound reduces background binding to normal tissues and improves the ratio of tumor to normal tissue localization of the carrier. This is of help in tumor imaging because a high tumor-to-background ratio is likely to allow resolution of smaller tumors and/or tumors with low uptake of targeted radionuclides. However, the therapeutic effectiveness of a given dose of conjugates is decreased because the proportion of the total administered amount of the $F(ab)_2$ moiety that localizes in a tumor is much less than that of the parent IgG.[17] The rapid clearance of labeled $F(ab')_2$ and F(ab) fragments from blood is likely to shift the equilibrium of binding, leading to the dissociation of the bound antibody away from the tumor. We have observed that MTX linked to the $F(ab')_2$ moiety of an anti-EL4 lymphoma IgG was a less effective tumor inhibitor than equivalent amounts of MTX linked to the parent IgG.[299]

Since an antibody-cell-surface TAA interaction can be regarded as a monovalent reaction,[289] it is not surprising that IgG antibodies and their F(ab) and $F(ab')_2$ fragments have nearly identical affinity constants.[293] However, monovalent carriers like the F(ab) fragment will be less susceptible to endocytosis or shedding after binding to the tumor cell surface because monovalent fragments do not cap. Although lack of shedding or endocytosis may improve the imaging quality of antibody-linked radionuclides and the therapeutic effectiveness of conjugated cell-surface active agents, it will render targeting ineffective for agents that interact with intracellular targets.

8. Dosage of Administered Conjugated Antibodies

If a cell surface TAA is freely exposed to circulating blood (e.g., as with leukemic cells), the amount of a conjugate that would bind to tumor cells will depend upon the concentration of the labeled antibody, the number of TAA sites available for binding, the affinity of the antibody, the amount of competing antibody (either in the conjugate preparation, or in the form of autologous anti-TAA antibodies), and circulating antigen. However, with "solid" tumors, additional factors will be the fraction of the cardiac output that reaches a tumor (usually small), the extent of the tumor bed to be supplied by the blood delivered, the capacity of the conjugate to cross the capillary wall, its rate of diffusion and stability in the extravascular compartment (especially in the acidic milieu),[277] and the high content of proteolytic enzymes that are usual in solid tumors. It has been recently reported that in melanoma patients given ^{111}In-labeled MAB 96.5 (directed against the P97 melanoma antigen) the number of tumor sites imaged increased with the amount of antibody administered.[140] It is possible that increasing the total antibody dose will saturate nontarget sites and thus further increase the concentration of the MAB in the milieu of tumor.

9. Pharmacokinetics

Even very mild treatment of antibodies during conjugation may alter their pharmacokinetics in vivo and reduce their circulating half-life as a result of rapid elimination of denatured antibodies.[300] Also, substantial uptake and catabolism of denatured conjugates by phagocytic cells could contribute to nonspecific localization. Another cause of nonspecific localization and rapid elimination of antibody agents is the presence of TAA or antigenically related materials in the circulation. There is usually an elevation in the serum level of many of the TAA and tissue-specific antigens (e.g., prostate-specific antigens) in cancer patients.

E. The Rationale and Limitations of Cancer Therapy with Antibody-Targeted Agents

These have been discussed before.[12,15] The rationale for the use of MAB as carriers of cytotoxic drugs in treatment is based on the demonstration that intravenously administered anti-TAA antibody shows target-selective in vivo localization.

1. Tumor Localization of Anti-TAA Antibodies

The evidence for tumor-specific localization of intravenously administered antibodies and the factors that determine the amount that reaches the tumor and its duration of sojourn will be briefly summarized. In evaluating the tumor-specific localization of anti-TAA antibodies, it should be remembered that considerable accretion of intravascularly administered macromolecules can occur in a tumor as a result of increased tumor vasculature, increased permeability of newly formed vessels, and the lack of lymphatics in tumors.[139,225] A variety of appropriately radiolabeled macromolecular carriers including serum albumin have successfully imaged a sizable proportion of human tumors due to intratumoral vascular and hemodynamic changes (i.e., sluggish flow of blood, increased blood volume, and the increased permeability of vessels inside tumors). Therefore, to demonstrate tumor-specific localization of anti-TAA antibodies, it is not only necessary to show higher localization of the labeled antibody in tumors compared to its localization in normal tissues, but also higher intratumoral localization of the labeled antibody compared to an immunologically irrelevant antibody of the same class and subclass. In one approach to establish tumor-specific localization of anti-TAA antibodies, anti-TAA and nonspecific Ig labeled with different radioiodine isotopes have been used, i.e., the so-called "paired label method".[300] Using these stringent criteria, the tumor-specific localization of both polyclonal and monoclonal anti-TAA antibodies and their immunologically reactive fragments have been established in experimental tumors including leukemia/lymphoma,[225,274,301-303] in human tumor xenografts,[139,304,305] and in cancer patients.[142,143,147,225] For example, using an ^{131}I-labeled MAB against melanoma TAA, Ghose et al.[139] and others[306] have achieved a several-fold increase in localization in tumors compared to other tissues. Specific tumor localization of MTX linked to anti-EL4-IgG (AELG) has been demonstrated by Ghose et al.[307]

For effective eradication of tumor cells by antibody-linked cytotoxic agents, it is essential that a sizable proportion of the administered antibody localizes and remains in the target tumor. However, experiments in the authors' laboratory showed that only approximately 0.55 to 0.65% of the administered activity of a ^{131}I-labeled antimelanoma MAB localized in human melanoma xenografts in nude mice at 48 hr; at 126 hr the proportion was still less, i.e., 0.27 to 0.56%.[17] With purified ^{131}I-labeled goat anti-CEA IgG, Mach et al.[147] have observed that while the concentration of the antibody in the tumor could reach ninefold higher than in the liver only 0.05 to 0.2% of the injected radioactivity was found in the resected tumors 3 to 8 days after injection. However in other tumors, uptake of MAB-bound radionuclides was much higher, e.g., 5 to 10% of the administered dose in the tumorous spleens of erythroleukemic mice,[308,309] 6.5% of the administered dose per gram in s.c. transplants of Thy 1.1 positive mouse lymphoma,[310] up to 20% of the total body activity in human tumor-xenograft-bearing nude mice,[311] and 17.2% of the administered dose in human melanomas (0.04% per gram of tumor tissue) after injection with the ^{131}I-labeled F(ab) fragment of anti-p97 MAB.[143] The interval between injection and the observed maximum was as short as 6 hr in erythroleukemic mice.[309] In Thy 1.1 positive s.c. transplants of a mouse lymphoma, the level of antibody rose in tumors over the first 24 hr and was maintained at this level for approximately 24 hr. The level declined exponentially thereafter ($t_{1/2}$ 104 hr), with a mean of 3% per gram remaining at 8 days. In contrast, only 1.5% per gram of a control antibody was present at 24 hr and remained in the tumor at that level over

8 days.[310] It is possible that the proportion of antibody that localizes in tumors can be increased by using a cocktail of antibodies against different TAA or different epitopes of the same TAA. DeLand and Goldenberg[93] have reported that a mixture of three MAB against GW39 did not produce a greater concentration of activity in the tumor than administration of a single MAB. However, it was not stated whether these three MAB were directed against the same epitope.

High affinity antibodies are likely to remain localized in tumors longer than antibodies with low affinity. It is therefore encouraging that the $F(ab')_2$ and F(ab) fragments of anti-TAA antibodies retain the affinity of the parent IgG molecule.[93,142] Additional factors that are likely to influence the proportion of antibody that localizes in tumors and the kinetics of localization include: (1) tumor size and weight (a direct relationship between tumor size and uptake has been attributed to probable nonspecific trapping);[311] (2) tumor vascularity; and (3) catabolism of the conjugate at the tumor site and removal of catabolized fragments. Catabolism of tumor-bound antibody has been observed between 6 to 24 hr of injection and has been found to be independent of Ig class and reactive fragment used.[309] Tumor-bound antibody may be released as a result of shedding of the bound antigen, endocytosis followed by intracellular catabolism, proteolytic activity on and around tumor cells, and normal turnover of TAA and membrane-associated glycoproteins. Major limitations of antibody-targeted drugs in the treatment of cancer include the following.

2. Circulating Antigens, Immune Complexes, and Blocking

In two studies on MAB therapy of leukemia, circulating antigen effectively blocked binding of the MAB to target cells in vivo.[312,313] However, blocking was overcome after repeated infusions in the study of Miller et al.[312] and not observed in several other serotherapy trials.[118,314] In melanoma patients with elevated serum levels of P97 antigen, lesions could not be imaged with ^{111}In-labeled MAB against the P97 antigen.[140] There are other reports showing that high serum levels of TAA such as CEA or hCG do not appreciably interfere with tumor localization of administered antibodies.[93,146] So far, the presence of a blocking factor does not appear to be predictable. However, the nature and cell surface distribution of an antigen may be a fair indicator of the possible presence of TAA in the circulation (see below). The amount of circulatory antigen appears to be related to tumor cell burden, and therefore circulatory blocking factors may not be a major factor if the tumor burden is reduced first by other modalities of therapy. Furthermore, elicitation of antibody responses to the carrier xenoantibody[141] and/or linked agents, i.e., drugs and protein toxins,[315] may limit the usefulness of conjugates.

3. Antigenic Modulation

Several studies have revealed antigenic modulation in target tumor cells both in vitro and in vivo.[118,316] However, these cells were able to reexpress the antigen when antibody was no longer present. Furthermore, antigenic modulation occurred only with certain cell surface antigens such as CALLA and surface Ig, but not with others, such as Ia and histocompatibility antigens. There appear to be several different mechanisms of antigenic modulation.[317] These include rearrangement of the antigen in the cell membrane (e.g., TL antigen), shedding of surface antigens (e.g., some MAA), and internalization of antigen, either alone[186] or antibody bound. Examples of the latter are the CALLA and EL4 mouse lymphoma antigens.[297] Proper scheduling of antibody infusions to allow reexpression of the antigen should overcome this problem. It may also be possible to select a MAB against an antigen that does not modulate.[118] Antigens such as CALLA or the prostatic P54 antigen[186] that internalize along with bound an-

tibody may be ideal targets for MAB-linked agents if the target molecules of the conjugate are intracellular.

4. Tumor Cells in Immunological Sanctuaries

Tumor cells in immunological sanctuaries such as the central nervous system may not be accessible to infused antibody conjugates. However, Ghose et al. demonstrated that the $F(ab')_2$ fragment of antitumor IgG can cross the blood brain barrier and selectively localize in intracerebral tumors.[16,264]

5. Antigenic Heterogeneity of Tumor Cells

Most, if not all, human tumors investigated have proven to be antigenically heterogeneous, i.e., 100% of any given histologic type of tumor does not react with a given anti-TAA antibody. Within a given tumor, cells show a wide variation in their reactivity to a given anti-TAA antibody and usually a proportion of cells does not have detectable TAA. Foulds[318] reported morphological heterogeneity in different areas of a single mammary tumor. More recently, the results of the reaction of 39 different human mammary carcinomas with 4 anti-TAA MAB have been discussed by Schlom et al.[173] Ten antigenic phenotypes emerged, ranging from those that express all four antigens to those that express none. There were also wide variations in the expression of TAA by different cells within a given tumor. One area of a tumor contained cells that expressed a particular TAA but in another area of the same tumor, cells lacked this TAA. TAA positive cells were found directly adjacent to TAA negative cells even in a given area. The TAA distribution in a positive cell population varied, i.e., TAA could be found focally in the cytoplasm, diffusely throughout the cytoplasm, or in the luminal borders of cells. Similar antigenic heterogeneity has been observed in most human cancers investigated, e.g., lung cancers,[212,319] melanoma,[320,321] prostatic carcinoma,[181] and others. Heterogeneity of tumor cells has been observed even after cloning.[280] Some MAB react with a proportion of cells in the primary tumor but with none in one or more metastatic lesions. For antibody-linked agents to be therapeutically effective, the reactivity of tumor cells with the carrier antibody should be closely monitored and, in certain cases, a cocktail of antibodies that react with all cells in a given tumor may be useful. It has been claimed[173] that biologic response modifiers such as interferon can induce the expression of TAA in antigen-negative tumor cell populations. Another approach to overcome antigenic heterogeneity of tumor cells, especially "patch-work" heterogeneity, is the use of appropriate radionuclides (instead of chemotherapeutic agents and protein toxins) so that the tumoricidal dose of radiation can extend up to several cell diameters.[15]

6. Relative Lack of Tumor-Specificity of Anti-TAA Antibodies

The lack of absolute tumor specificity of available MAB also remains a formidable problem. For example, CALLA is expressed not only by non-T-cell ALL (80% of patients), blast cells in CML crisis (40% of patients), and a variety of lymphomas, but also by a small proportion of normal bone marrow cells. More pertinently, in most of the clinical studies on the serotherapy of T-cell ALL, T-cell lymphoma, and B-cell CLL, the MAB used not only reacted with tumor cells but also with T-cells in peripheral blood, spleen, lymph nodes, and thymus.[116] Effective tumor suppression also led to immunosuppression. Furthermore, the presence of large numbers of non-neoplastic antigen-bearing cells would interfere with the binding of the passively administered antisera to tumor cells and would require infusions of larger amounts of xenoglobulins. Unfortunately, MAB that appear to be more specific for tumor cells are directed against antigens that are only expressed in the tumors of a small proportion of patients (e.g., MAB 89 against a human lymphoma-associated antigen,[322] MAB 4C7 and 3C2

against ovarian carcinomas,[323] MAB against non-SCLC lung tumors,[324] a MAB against colorectal carcinomas,[155] and MAB against breast carcinomas[172]). The idiotypic configuration of the B-cell surface Ig furnishes what is, at present, probably the most specific TAA. With a cytotoxic agent linked to an anti-idiotypic antibody, the only normal cell population that would be destroyed would be those B-cells that have the same idiotype as the tumors. Antibodies directed against the idiotype configuration of T-cell receptors, when available, are likely to have the same degree of specificity. Although inhibition (or eradication) of non-vital normal cell populations (e.g., prostatic tissue and endocrine and exocrine glands) may not always be a serious limitation, MAB of greater tumor specificity should be sought.

7. Emergence of Resistant Tumor Cell Populations

It is possible that tumor cell populations that lack the targeted TAA or are resistant to the cytotoxic agent will emerge after repeated administrations of antibody-linked agents. Ghose et al.[128] and others[118,316] have observed the emergence of antigen-negative tumor cell populations either as a result of modulation or of immunoselection of antigen-negative tumor cell variants after prolonged administration of antibody, either alone (leukemia patients) or linked to chemotherapeutic agents (melanoma patients). Use of carrier antibodies directed against different TAA and the use of alternate chemotherapeutic agents that exert a cytotoxic effect on the resistant tumor cell population may overcome this problem. Fortunately, methods are now available for binding different classes of cancer chemotherapeutic agents and a variety of protein toxins for the production of therapeutically effective conjugates.[14,18]

8. Lack of Vascularity in Solid Tumors

It is unlikely that systemically administered anti-TAA antibodies would reach avascular and necrotic areas in "solid" tumors. In fact, Ghose et al. failed to observe any localization of the labeled antibody in necrotic areas in tumors or in necrotic tumors in patients with primary or metastatic renal carcinoma given ^{131}I-labeled polyclonal antirenal carcinoma IgG.[225] It should be emphasized that surgery and/or radiation are likely to remain the effective and preferred methods for the treatment of localized tumor masses. At present, chemotherapy alone is used in the treatment of disseminated tumor cells. It is hoped that the use of an antibody-linked cytotoxic agent will increase the therapeutic index of that agent, i.e., render it more tumor selective. Furthermore, the additive and occasionally synergistic antitumor actions of anti-TAA antibodies and chemotherapeutic agents may add to cytocidal action.[12] The cytocidal action of most chemotherapeutic agents follows first-order kinetics (rendering the probability of the eradication of the last tumor cell low), whereas the action of anti-TAA antibodies alone or agent-linked antibodies follow zero-order kinetics. Therefore, antibody-linked cytotoxic agents are likely to be more effective in eradicating circulating tumor cells and microemboli than unconjugated agents.[12] Thus, this modality of therapy is likely to be most effective in the eradication of residual disseminated cells, micrometastases, and leukemias after reduction of tumor mass by other modalities of therapy.[29] Furthermore, large tumor burdens are also likely to be associated with elevated levels of circulating TAA that would "neutralize" antibody-linked agents.

9. Toxicity and Detrimental Effects of Antibody-Linked Agents

Untoward effects of agent-antibody conjugates may be due to either components, as well as to contaminants such as pyrogen. Adoption of stringent aseptic procedures and routine testing of preparations for pyrogen virtually abolished the risk of pyrogen-induced reactions.[225,276] Adverse effects due to antibodies include the following. There may be reactivity of the carrier antibody with nontarget cells due to a lack of absolute

tumor specificity of the carrier antibody, e.g., the systemic toxicity of an anti-CEA antibody that cross-reacted with circulating granulocytes and erythrocytes,[92] or the suppression of immunological reactivity as a result of the cross-reactivity of antilymphocytic leukemia antibody with immunocompetent cells.[118] Foreign Ig, as well as the ligands, are likely to be immunogenic and, after repeated administrations, may provoke an immediate hypersensitivity reaction[276] or immune complex-mediated reactions due to the production of precipitating antibodies against xenoglobulins. Such antibodies are also likely to interfere with the tumor-specific localization of conjugates and divert conjugate-containing immune complexes to organs rich in reticuloendothelial cells such as the liver and spleen.[92] However, after removal of aggregated Ig from chlorambucil-antimelanoma IgG conjugates, Ghose et al. failed to detect any precipitating antibody in patients given repeated injections of chlorambucil-polyclonal IgG.[276] Removal of Ig aggregates and initial high doses of anti-TAA Ig may induce tolerance instead of provoking a humoral response. Administration of murine hybridoma-derived MAB to patients with lymphoproliferative disorders[116,312,325-327] or mammary carcinoma[173] had very few ill effects. However, occasional adverse effects after infusion of MAB have been reported.[317] In the trial conducted by Miller et al.[328] on the effect of MAB anti-Leu 1 in T-cell lymphoma, antibodies to mouse Ig appeared in four of seven patients and contributed to the loss of effect of the antibody in three patients. Dillman et al.[329] attributed the lack of effectiveness of MAB T101 in two of four cutaneous T-lymphoma patients to the development of antimouse Ig antibodies. Sears et al.[330] could detect human antimouse Ig antibodies in 9 of 18 gastrointestinal carcinoma patients receiving MAB 1083-17-1A, but no adverse effects were noted.[312,325-327,331] In an extensive study, Schroff et al.[141] assayed the serum level of antimouse Ig in CLL, cutaneous T-cell lymphoma, and malignant melanoma patients. Elevated serum levels of antimouse Ig were seen in 0 of 11 of the leukemic patients and 3 of 9 of the melanoma patients. In one melanoma patient, the antibody appeared to be anti-idiotypic. No clinical toxicity was observed.

Immunologically reactive fragments of anti-TAA antibodies from which the Fc moiety has been removed may be less immunogenic. The availability of anti-TAA MAB of human origin is also likely to reduce immunogenicity. It is possible that recombinant DNA technology will provide human MAB after the isolation and linkage of genes coding for the antigen-binding region and the rest of the Ig molecule.[173] In addition to routine assay of serum for the detection of antibody against xenoglobulins, patients who are being given repeated injections of antibody-linked agents should be monitored by skin testing for their reactivity to conjugates during the administration of agents.[276]

Cytotoxic agents in conjugates may also cause adverse effects. After the linkage of low molecular weight cancer chemotherapeutic agents to Ig, there are likely to be changes in the toxicity and pharmacokinetic properties of the linked agent. For example, the rate of clearance of MTX from the circulation is considerably reduced with concomitant increase in its toxicity when the drug is administered as an IgG-conjugate.[307] On the other hand, the toxicity of several other cancer chemotherapeutic agents is reduced after linkage to macromolecular carriers, e.g., adriamycin and Trenimon.[13] However, cleavage of such agents from the carrier either in circulation or after intracellular catabolism may enhance the toxicity of the preparation. This is especially pertinent when conjugates containing both the A and B chains of protein toxins are used. Even when conjugates contain only the A chain, phagocytosis or pinocytosis of conjugates by non-target cells can contribute to systemic toxicity and lethality.[332]

IV. METHODS OF LINKAGE OF CYTOTOXIC AGENTS TO ANTIBODIES

Reviews referred to in the introduction include descriptions of binding methods.[13,14,16] Here, a summary of general principles highlighting major methods along with selected examples will be given.

A. General Principles

1. Preservation of Antibody Activity

Preservation of the activity and affinity of the carrier antibody is essential but may be difficult to achieve in practice. A linkage procedure that takes place under mild conditions is desirable since it appears that Ig, especially MAB, vary widely in their susceptibility to denaturing conditions. Even very slight denaturation of antibodies during labeling may alter their pharmacokinetics in vivo, e.g., the half-life of circulating antibodies has been observed to be considerably shortened after labeling with ^{131}I.[300] The presence of the bound agent may also alter the conformation of the combining site, especially if the agent is distinctly hydrophobic or possesses multiple charged groups. Agents, particularly proteins, may produce steric hindrance of antigen binding. Substitution may occur in the antigen-combining site of the Ig and prevent access by the antigen if the reactive functional group being used for the conjugation reaction is present in that site as well as in other regions of the Ig. These effects are likely to become more pronounced as the incorporation ratio increases. For example, when agents such as chlorambucil, MTX, adriamycin, daunorubicin, etc. are directly linked to Ig, loss of antibody activity can be substantial when the molar incorporation ratio of drug to antibody exceeds 10.[14] According to Pressman,[300] radioiodination at the level of less than 2 atoms of iodine per IgG molecule did not affect the activity of tumor-localizing antibodies, but localizing activity was reduced to 30% of the original when 19 atoms of iodine were present. In comparing the use of reactive fragments instead of the intact Ig molecule, Ghose et al. observed that that $F(ab')_2$ moiety of a rabbit anti-BSA IgG lost antibody activity at lower levels of incorporation of MTX compared to the parent IgG.[299] Measures may be adopted to protect the antigen-binding sites. An example is immobilization of the antibody on antigen-containing matrices during conjugation procedures.[300] The choice of linkage groups in the Ig (e.g., carbohydrates or SH groups) that are likely to be absent from the antigen-binding site may also promote retention of antibody activity.[14,16]

2. Preservation of the Activity of the Cytotoxic Agent

The activity of a cytotoxic agent in conjugate form must be considered in relation to the in vivo disposition of the conjugate. Either the activity must be preserved in the intact conjugate or provision must be made for the release of an active cytotoxic agent at the target site. If the conjugated agent can ultimately be released at the target site in an active form (but not necessarily its original form), there is no restriction on its interim chemical manipulation associated with the linkage reaction. In this context, the linkage reaction must either be reversible or susceptible to metabolic action which, in effect, generates an active agent. If the agent is to act on its target site in conjugated form, either intact or as a metabolic fragment, then groups in the agent essential for its action must not be used for linkage and must not be sterically hindered by other groups in the molecule involved in the linking bond. Also, retention of activity is more likely if a nonessential group is linked via a spacer arm to the Ig since steric considerations may be paramount.

3. Pharmacokinetic Considerations

A conjugate must be stable in transit via the circulation and extracellular space to target sites. The linkage reaction must neither be too readily reversible nor inappropriately susceptible to enzymatic disruption or the agent is liable to be released from the carrier prior to reaching the target cell. Aside from this consideration, in many cases chemical linkage designed to appropriately release the bound agent in vivo would constitute the ideal method. However, restrictions on the functional groups available in both agent and Ig limit such design.

High molar incorporation of drugs into Ig with the use of spacers and intermediaries or the linkage of large protein toxin molecules to antibodies may substantially alter the size and charge of the carrier antibody. This can impede its transcapillary passage and diffusion in tissue space and ultimately alter its pharmacokinetics. Furthermore, large and/or negatively charged conjugates are likely to be rapidly cleared from the circulation by phagocytic cells of the reticuloendothelial system and thus diverted from the target tumor tissue.

4. Functional Groups Used for Binding

Binding methods depend on groups that are present or can be introduced into the agent and Ig and the number of reactive groups in Ig limits the number of molecules of agent that can be attached. Reactive groups in Ig potentially utilizable for linkage occur in the side chains of several of the 20 amino acids as well as in the carbohydrate moieties.[14,18] They include aliphatic carboxyl, amino, disulfide, and hydroxyl groups; imidazole and phenolic rings; aromatic hydroxyl groups; and vicinal diols. The most widely used thus far have been carboxyl and amino groups. Introduction of reactive functional groups into Ig is exemplified by a number of variations in approach to linking through sulfur-containing bridge moieties based on the use of heterobifunctional reagents.[14,18] These have been applied most frequently to conjugating protein agents, e.g., toxins, so they will be discussed below under that heading. The production of F(ab)-SH by reductive splitting of IgG also represents, in effect, the incorporation of an -SH group.

Functional groups in agents also require careful consideration.[14,18] Those in protein agents are essentially the same as outlined for Ig, so here low molecular weight chemotherapeutic agents will be considered. The carboxyl group has often been used because it allows mild coupling reactions. If more than one is present in a drug, e.g., MTX, there is the possibility of activation of additional groups leading to cross-linking. For this reason, it may be desirable to limit the molar ratio of reagent to drug during the activation stage or utilize a regiospecific method. Other groups of importance, because of convenient linkage chemistry, include the amino group, the hydroxyl group, and vicinal dihydroxyls. Incorporation of reactive functional groups into drugs may also be feasible. However, the structural chemistry of drugs and their derivatives is outside the scope of this review, so the literature on individual drugs should be consulted; only a few examples pertinent to conjugate design are included here. Carboxyl groups can be introduced by reaction of an existing amino group with an anhydride. An interesting example of this is the use of *cis*-aconitic anhydride to produce a carboxy derivative which is susceptible to hydrolytic decomposition under mildly acid conditions, such as exist in lysosomes.[333] Succinic anhydride forms an analogous, more stable derivative.[334] Introducing a carboxyl group in this way also has the effect of introducing a potential spacer and this aspect is discussed below. A derivative of daunorubicin can be obtained in which the methylketone side chain is modified to the corresponding 14-bromomethyl ketone. The activated halogen atom alpha to the carbonyl group is capable of reaction with protein amino (and carboxyl) groups. Zunino et al.[335] prepared stable covalent conjugates with a number of proteins by incubating

14-bromodaunorubicin with the protein at pH 8.5. Molar incorporation ratios ranged from 0.1 (lysozyme) to 3.7 (BSA) and 8.5 (Ig).

B. Outline of Binding Methods

Important linkages utilized for conjugation include amides, disulfide bridges, and Schiff base-derived groups. Stabilities in vivo can range widely and are likely to be influenced strongly by steric factors arising from the juxtaposition of agent and Ig moieties. Representatives at the low to moderate end of the scale include esters and amides that are susceptible to lysosomal hydrolases. Disulfide bridges are likely to be susceptible to reductive splitting, whereas the thioether bond would be expected to be more stable. The behavior of conjugates based on linkage groups such as aziridyl amides, hydrazones, and Schiff bases will be influenced by their chemical lability. This could be of value as long as the agent was not released prior to reaching the target tissue. An aziridyl amide is relatively stable at the pH of blood but is hydrolyzed at pH 4 with a half-life of a few hours so breakdown in lysosomes should be promoted.[336] In this context, various lysosomotropic conjugates[337-339] have been designed and some are discussed below. When spacers or intermediaries are introduced, there can be several linkage bonds between the agent and the Ig, and the properties of these multiple groupings may be important in the manifestation of a cytotoxic action. A dramatic example is the difference in cytotoxicity between the *cis* aconityl and maleyl derivatives of daunorubicin. The most labile link will govern in vivo splitting of the conjugate into a "protein fragment" and an "agent fragment".

1. Noncovalent Binding

Noncovalent binding directly between agent and antibody has not been generally applicable. Parameters involved have been discussed by Edwards[340] and by Ghose et al.[14] One noncovalent-binding system that has potentially more general applicability to conjugate synthesis is avidin-biotin (see Ghose et al.[14]). The methodology entails linking the agent to avidin and the Ig to biotin, or vice versa, followed by combination of the two binary conjugates. Successful conjugation depends on not blocking either the avidin or the Ig-binding sites. Although the binding between avidin and biotin is noncovalent, the dissociation constant is of the order of 10^{-15}.[341] This is indicative of very high stability, and a combination of low pH and denaturing agents or proteolysis would be required to split a conjugated agent. The analog, 2-imminobiotin, forms a strong interaction at high pH values, but dissociation is easier at low pH where it becomes positively charged.[341]

The avidin-biotin interaction has been applied recently to toxin-antibody conjugation.[342] Hashimoto et al.[342] reacted the NHS ester of biotin with IgG or $F(ab')_2$ in 0.1 *M* sodium bicarbonate to form the Ig-biotin derivative. Linkage of the agent to avidin was achieved by disulfide interchange since the technique was being applied to a toxin A chain. Avidin was reacted at pH 7.6 with a fivefold molar excess of SPDP to obtain the PDT derivative (PDT-avidin). Toxin A chain bearing a free -SH group was prepared by reduction with a low molecular weight sulfhydryl compound and allowed to react with PDT-avidin to produce "A-chain-S-S-avidin". The product was purified by chromatography on Sephadex® G-150 to yield mainly the 1:1 binary conjugate. Incubation of the two binary conjugates in buffer formed the noncovalent ternary conjugate. In an alternative approach, Hashimoto et al.[342] exposed target cells to biotinylated Ig followed by the avidin-bearing agent.

Another approach to noncovalent binding is to utilize the antibody-antigen interaction where high affinities can be observed. The agent has been used as antigen so that antigen-antibody complexes can be targeted to Fc receptor-bearing cells.[343] Alternatively, hybrid antibodies have been synthesized that combine target-antigen-binding

and cytotoxic-agent-binding moieties by oxidative combination of the appropriate F(ab) -SH fragments.[344] However, endocytic uptake by cells may be less than expected for a bivalent antibody if the hybrid is univalent with respect to binding to a cell surface target antigen. On the other hand, the hybrid may serve to deliver agent to a cell surface-binding site for that agent if one is present.

2. Covalent Linkage

a. Direct Linkage between Agent and Antibody

The simplest approach to direct linkage entails a single incubation of agent and Ig along with an activating reagent. The most commonly used such single-stage method has been the formation of amide bonds by use of a water-soluble carbodiimide. This method can be applied to a carboxyl group-containing drugs, such as chlorambucil or MTX, or to a protein agent. Its chief advantage lies in the mild conditions that can be used to achieve coupling, while its chief disadvantage arises from the simultaneous presence of carboxyl and amino groups in proteins. This has the potential for producing cross-linking that may be difficult to control. (The presence of reactive carboxyl and amino groups in the agent can be similarly disadvantageous.) Attempts have been made to control cross-linking by first allowing the carbodiimide to react with the carboxy-containing compound and then introducing the Ig. In preparing daunorubicin-IgG conjugates, Gallego et al.[334] were able to restrict intermolecular cross-linking of IgG to less than 5% (estimated by SDS-PAGE) by using a molar ratio of 1:25:50 for IgG, drug, and ECDI, respectively.

Other conjugation methods have been developed that avoid cross-linkage and homopolymerization. Some involve preparing monofunctional derivatives of the agent or the Ig that are sufficiently reactive to become coupled under the mild aqueous conditions required to prevent denaturation of protein. The strategy is illustrated by the synthesis of active ester derivatives of carboxyl group-containing compounds that do not also possess an unprotected reactive nucleophilic group. The free carboxyl group can be derivatized by reaction with NHS and a carbodiimide. In the second stage, the active ester will react under mild aqueous conditions with nucleophilic groups in the Ig, chiefly amino groups, to produce amide linkages. This method has been applied to MTX conjugation.[346]

Another way of activating a carboxyl group-containing drug is to produce a hydrazide by reaction of hydrazine with either an ester or active ester derivative of the drug. Hydrazide derivatives will react with aldehyde groups to form hydrazones. This has been done with a fraction of the aldehyde groups in the intermediary, polyaldehyde dextran.[346,347] The drug-carrying polyaldehyde dextran was then allowed to react via remaining aldehyde groups with amino groups in an Ig. However, this latter step reintroduces the risk of cross-linking due to more than one Ig molecule reacting with the multiple aldehyde groups present on a given dextran molecule. Alternatively, a hydrazide derivative may be converted to the corresponding azide by reaction with nitrous acid. The azide will react with amino groups in proteins or in polylysine as intermediary. Various homo- and heterobifunctional reagents have also been used for synthesis of antibody conjugates, especially in the linking of other proteins to Ig.[14,18]

b. Linkage through Spacer Arms

Steric hindrance must be prevented if a conjugated agent is to interact with its molecular target while the agent is still bound to Ig. The same approach can be applied to linkage of agents to antibodies, as is used in affinity chromatography to overcome steric hindrance. In situations where the agent is released prior to exerting its cytotoxic effect, a moiety equivalent to a spacer may still be introduced so as to allow linkage bonds of a particular kind, e.g., pH-sensitive.[337,348]

Examples of lysosomotropic spacers are furnished by a series of glycopeptide derivatives of daunorubicin. Monsigny et al.[338] synthesized 2-(1-thio-beta-D-glucopyranosyl)-ethanoyl-L-arginyl-L-leucine (Glc-S-Et-Arg-Leu), the choice of arginine being based on specificity of lysosomal proteases and of leucine on the fact that leucyl daunorubicin is fully active. This intermediate (bearing a free carboxyl group on the leucine moiety) was reacted with NHS and ECDI to form the corresponding active ester which, in turn, was reacted with daunorubicin via the drug amino group to produce a binary amide-coupled drug-spacer conjugate. The sugar ring was then oxidized with periodate to yield a dialdehyde which is capable of coupling to proteins (in this case wheat germ agglutinin) through Schiff base formation. Final stabilization was achieved by reaction with borohydride. Incorporation ratios were low since above 1.5 mol of drug per mole of protein the conjugate became insoluble. The ternary conjugate bound readily to lectin receptors at 4 and 37°C and was more effective than the free drug in inhibiting growth of L1210 cells. A specific competitive effect on both binding and cytotoxicity was observed with di-*N*-acetylchitobiose. Fluorescence microscopy showed membrane labeling after 30 min and fluorescence in clusters after 1 hr. Lysosomal protease susceptibility was shown by preparing solvent extracts of exposed cells and subjecting them to TLC. Derivatives with Rf values similar to free daunorubicin, to leucyldaunorubicin, and to the binary drug-spacer conjugate were observed. The binary conjugate, while taken up by cells slowly, was not cytotoxic and did not release free daunorubicin or a leucyl derivative.

This group later reported on a series of L-Ala-L-Leu peptides spacers with up to four residues, intended to be lysosomotropic.[339] Leu-, Ala-Leu-, Leu-Ala-Leu-, and Ala-Leu-Ala-Leu-daunorubicin were first synthesized. ECDI was used to conjugate succinylated BSA (sBSA) with the amino group of daunorubicin or its peptide derivative. Incorporation was 10 to 21 mol of daunorubicin per mole of sBSA. Succinylation of the protein to block reactive amino groups decreased the extent of polymer formation and about 70% of the conjugate could be isolated in the monomer fraction from Sephadex® 6B chromatography. Incubation of these conjugates with a rat liver lysosomal fraction at pH 5.5 showed that a spacer length (n) of three or four amino acid residues was required for release of drug as determined by HPLC. The linkage was stable in serum, a finding consistent with maintaining the integrity of conjugates in transit to target sites. An i.p. injection of 2 mg/kg of free drug on days 1 and 2 into L1210-cell-inoculated mice showed a 39% increase in life span. Injections of conjugates showed no prolongation when n = 0 or 1, an effect less than the free drug when n = 2, but prolongation of survival of the order of 200% when n = 3 or 4. Also, lower weight loss in the conjugate-treated group indicated a reduction in toxicity compared to the free drug.

A logical refinement of this conjugation procedure was to prepare the active ester of Ala-Leu-Ala-Leu-daunorubicin (and the corresponding adriamycin derivative) and react that with the protein instead of using carbodiimide coupling.[349] Reaction of the free amino group of the peptide with succinic anhydride was used to first introduce a carboxyl group; conversion to the active ester was then carried out with NHS. Conjugation was effected by reaction with protein at pH 9. Conjugates with serum albumin incorporated 10 to 12 mol of ligand per mole of albumin while conjugates with MAB IgG2B and IgG3 incorporated only 3 mol of drug per mole of IgG. The active ester concentration could not be increased because of precipitation. Susceptibility to denaturation may depend on the particular active ester as well as the protein since Kulkarni et al.[345] were able to achieve incorporation ratios of 10 mol/mol of IgG and 90% recoveries of protein using an active ester of MTX.

Shen and Ryser[333] made use of an interesting system for investigating potentially lysosomotropic conjugates. They prepared *N-cis*-aconityl and *N*-maleyl derivatives of

daunorubicin by reacting the drug with the respective acid anhydrides. When the *N-cis*-aconityl derivative was conjugated to an amino group-containing solid matrix, the half-life for hydrolysis was 3 hr at pH 4 and more than 96 hr at pH 6 or above. This conjugate had no effect on growth of WEHI cells at pH 7. When *N-cis*-aconityl daunorubicin was conjugated to poly-D-lysine using ECDI, the resulting binary conjugate could be taken up by WEHI cells and strongly inhibited growth. (The poly-D-lysine polymer backbone is stable intracellularly, unlike poly-L-lysine.) A binary conjugate of *N*-maleyl daunorubicin and poly-D-lysine could also be taken up by cultured WEHI cells but had no effect on growth, as expected since the maleyl group lacks the *cis*-carboxyl which is responsible for ease of hydrolysis. Analogous binary conjugates could be synthesized with MTX by coupling to poly-D-lysine with and without the digestible spacer, triglycine.[350]

c. Linkage through Multivalent Intermediaries

Limitations on the molar incorporation ratio achievable by direct linkage can be overcome by linkage via multivalent intermediaries such as dextran, polylysine, pGA, and serum albumin.[351] At equimolar incorporations, loss of activity is likely to be less when ligands are bound via an intermediary. However, this has not been systematically investigated. Even though the intermediary may incorporate a large number of substituent molecules, conjugation of a large drug load may alter the properties of the complex such that denaturation takes place.

i. Poly Amino Acids

Early attempts to form conjugates with pGA as the multivalent intermediary used ECDI both to link the drug to the pGA and to link the binary drug-pGA conjugate to Ig. *p*-Phenylenediamine mustard was linked in this way.[352] Later, Hara's group[353] took advantage of the fact that in the unique terminal carboxyl groups a pGA molecule can provide a single site of attachment to Ig. This avoids polymerization arising from attachment of multiple Ig molecules to the intermediary. One approach was first to use SPDP[354] to prepare monopyridyldithio-pGA (R-S-S-pGA) and treat it with DTT to reduce the R-S-S-pGA to the corresponding free -SH compound, HS-pGA. The mixture of pGA and HS-pGA was next treated with thiopropyl Sepharose 6B to bind the HS-pGA by disulfide formation and so allow removal of the unreacted pGA. The washed resin was treated with excess MET to release the HS-pGA by disulfide interchange which, finally, could be reconverted to R-S-S-pGA by reaction with an excess of 2-pyridyl disulfide. An alternative method for introducing the terminal sulfhydryl residue into pGA entailed polymerizing gamma-benzyl-*N*-carboxy-L-glutamate anhydride with cystamine to form a mixture of pGA-CO-CH_2-CH_2-S-S-CH_2-CH_2-CO-pGA and pGA.[355] Reduction with DTT then produced the free -SH compound which was reacted with thiopropyl Sepharose 6B as described above to remove pGA lacking an -SH group. Finally, reaction was carried out with an excess of 2-pyridyl disulfide to protect the thiol and allow for spectrophotometric assay. This formed R-S-S-CH_2-CH_2-pGA of an average molecular mass of 12 kdaltons.

Ternary conjugate formation using R-S-S-pGA to which an agent had been coupled (see below) was carried out as follows.[353] The Ig was substituted with maleimide groups by reacting it with a 20-fold molar excess of SMBE or of the corresponding butyrate, SMBU, which produced a more stable derivative. The average number of maleimide groups incorporated per molecule of Ig was 6.7 (SMBE) and 12.1 (SMBU). Finally, the drug-substituted R-S-S-pGA was reduced with DTT and reacted with the maleimide-substituted Ig to form the ternary conjugate. For daunorubicin conjugation, purification of the ternary conjugate was carried out by starch-block electrophoresis, which will remove contamination by drug-substituted R-S-S-pGA that has not reacted with

Ig. Incorporations of one to three drug-carrying intermediary molecules per molecule of Ig were obtained, depending on the molar ratio (4 to 10) of drug-substituted R-S-S-pGA to maleimide-substituted Ig in the reaction mixture and on the maleimide content of the Ig. Essentially, the same method was used for coupling of drug-substituted R-S-S-CH_2-CH_2-pGA to Ig.[355] The biological behavior of ternary daunorubicin-pGA-antibody conjugates is described under Section V, Current Status.

The method of linkage of a particular agent to pGA-bearing -SH groups in preparation for ternary conjugate formation will depend on the functional groups present in the agent. These workers coupled daunorubicin simply by using a water-soluble carbodiimide since R-S-S-pGA lacks an appropriate nucleophilic group and would not be expected to undergo polymerization. To allow ARA-C to be similarly linked using ECDI, an aminoalkylphosphoryl group was introduced into the drug at the 5′ hydroxyl of the sugar.[356] Linkage to pGA by ECDI was regiospecific and 20 to 30% of the gamma carboxyl groups were substituted. ARA-C was also linked at the 4-amino of the cytosine moiety by reaction with a mixed anhydride derivative of pGA to form an amide bond. In this case, 15 to 20% of the residues were substituted. With both types of conjugates, the remaining free carboxyl groups of pGA could be blocked with 2-aminoethanol and ECDI to convert them to the corresponding hydroxyethyl amides. The 4-amino-group-based linkage was slowly hydrolyzed in aqueous buffer at 37°C; 17% of the drug was released at pH 7.4 after 4 days and 36% at pH 5 after 4.4 days. The alkylamino-group-based linkage was stable in aqueous buffer but was 40% hydrolyzed by phosphodiesterase 1 after 24 hr at pH 7.4. In vitro cytotoxicity tests based on continuous exposure of L1210 cells for 70 hr gave the following order of effectiveness: free ARA-C >> 4-amino-based conjugate > aminoalkyl-based conjugate. Blockage of carboxyls in the aminoalkyl-based, but not in the 4-amino-based, conjugates reduced cytotoxicity. The authors explained these observations on the basis of hydrolytic release of free drug from the 4-amino-based conjugate.

Tumor inhibition was assayed after both single and repeated i.p. doses into tumor-inoculated animals. In contrast to the in vitro results, the conjugates were equal to or more effective in vivo than the free drug. The 4-amino-based conjugate in which the carboxyl charge was neutralized was the most effective, producing some long-term survival with the repeated dose schedule. The mode of in vivo action of these binary conjugates may involve both extra- and intracellular release of drug. Factors determining effectiveness would include "the type and stability of the chemical linkage binding drug to macromolecule, the length of spacer arm, and the nature of the macromolecule itself".[356] It will be interesting to study the properties of ternary conjugates with antitumor antibodies.

ii. Proteins

Use of a protein as a multivalent spacer may be considered in the same category as coupling of a protein agent to Ig, as is dealt with in a following section. The attractiveness lies in the presence in the spacer protein of multiple potential linkage groups, e.g., amino, carboxyl, etc. Serum albumin, the main protein used for this application, possesses desirable characteristics of stability in aqueous media over a fairly wide pH range and of tolerance toward organic solvents to some degree.[351] Incorporation of an agent into a spacer protein and the subsequent coupling of the binary conjugate to Ig may pose problems of cross-linkage and polymerization.

iii. Polysaccharides

Dextran has been used to link several agents to Ig. These include: daunorubicin,[357] bleomycin,[358] mitomycin-C,[359] MTX,[360] ARA-C,[361] FUR,[361] and adriamycin.[13] With most amino group-containing agents, the approach has been to first oxidize the dextran

with periodate to form polyaldehyde dextran. The polyaldehyde dextran is then incubated in aqueous solution with the drug for several hours, followed by the Ig for a further several hours or vice versa. This approach produces a composite Schiff base but leads to polymerization due to the presence of multiple amino groups in Ig. The Schiff bases can be finally treated with borohydride or cyanoborohydride to stabilize the linkage.

An alternative approach has been to utilize dextran hydrazide for coupling of an aldehyde derivative of the agent to Ig.[361] A procedure for preparing dextran hydrazide has been published by Hurwitz et al.,[347] and aldehyde groups can be introduced into agents that possess vicinal hydroxyl groups, e.g., nucleosides, by periodate oxidation. Coupling with dextran hydrazide has been effected by forming a hydrazone with the periodate-oxidized agent followed by linking of the binary hydrazone conjugate to Ig using 0.08% glutaraldehyde.[361] Although the actual reaction may be complex, one terminal aldehyde group of glutaraldehyde would be expected to form a hydrazone with the hydrazide groups of the dextran carrying the drug moiety. The other would form Michael adducts with amino groups in the protein as in typical glutaraldehyde cross-linking. In this synthesis, stabilization by reduction was not carried out.

Carbohydrate moieties in Ig constitute an intrinsic multivalent intermediary since periodate oxidation of the vicinal hydroxyl groups produces multiple aldehydes which can react with amino or hydrazide groups in an agent. However, when aldehyde groups are formed in the carbohydrate moieties of Ig, there occurs the possibility of forming intra- and intermolecular cross-links depending on steric factors. Hemiacetals may form with hydroxyl groups; aldol condensation may occur with another aldehyde group; imines may form by reaction with amino groups in the same or another Ig molecule. In addition to the cross-linking, these reactions can interfere with conjugation of an amino or hydrazide derivative of the agent. However, some of the aldehyde side reactions will be reversible and thus overcome by an excess of agent in the coupling stage. Certain of these considerations also apply to polysaccharide intermediaries.

d. Linkage of Protein Agents to Immunoglobulins

Methods for protein-protein conjugation have been recently and extensively reviewed.[14,18,340,344,362-366] The article by Blair and Ghose[18] lists most types of reactions along with relevant equations. Detailed directions for preparing toxin-antibody conjugates along with important information on safety precautions have been given by Cumber et al.[367] and Domingo and Trowbridge.[368] Here, we will comment briefly on current methodology.

An advance over simple homobifunctional reagents has been to employ heterobifunctional reagents in which two different functional groups possess differential reactivities that can be exploited in conjugation. This provides the ability to substitute a reagent molecule into protein 1 (e.g., the antibody) with the other functional group remaining intact. Ideally, there should be a high yield of the monomeric derivatized protein 1 that can then be coupled with protein 2 (e.g., the toxin). One may also consider this approach in terms of chemical modification of one or both proteins so as to introduce suitable reactive groups. The second stage in conjugation is then carried out under another set of conditions that favor reaction of the newly introduced functional group(s). Depending on the method, the product distribution can be influenced by the concentration ratio of the modified protein 1 and protein 2 if the latter contains more than one reactive linkage site. Keeping the second protein in excess will favor formation of 1:1 conjugates rather than formation of conjugates consisting of two antibody molecules, one toxin molecule, and two or more antibody molecules, etc. Conditions can be chosen to minimize these unwanted products and they can be excluded more definitively by ensuring that protein 2 also has only one site or group that will react

with the substituted protein 1, as is the case with a reduced toxin A-chain-SH or reduced Fab-SH.

An example of a heterobifunctional reagent is the NHS ester of chlorambucil.[364] The *N*-hydroxysuccinimidyl moiety will react with amino groups of protein 1 at low temperature, and then after purification of the derivatized protein, the introduced alkylating moiety will react with amino groups of protein 2 at higher temperatures. If six to eight chlorambucil molecules are introduced into one Ig molecule, then reaction with a four- to fivefold molar excess of a second protein will yield 5 to 10% of a 1:1 conjugate.[367] Another example of the heterobifunctional reagent approach is the use of the NHS ester of *N* (4-carboxycyclohexylmethyl)-maleimide to introduce maleimide groups into a protein which can then react with thiol groups of a second protein such as reduced Ig or F(ab)-SH.[369] As described above, SMBE or SMBU can be used to incorporate maleimide groups into Ig which can subsequently react with a second molecule carrying a thiol substituent to form a ternary conjugate.[353]

SPDP has become widely used to introduce a disulfide group directly into proteins[354] and Ghose et al. have outlined its use in production of antibody conjugates.[14,18] The group introduced is PDT and its extent of substitution may readily be determined by spectophotometry. Depending on the protein and reaction conditions, a sixfold molar excess of SPDP can result in the substitution of about three groups per protein molecule.[354,368] The PDT group is susceptible to a disulfide interchange reaction with another protein possessing a free -SH group. The final product will be a conjugate with a disulfide bridge linking the two proteins. The second protein with a free -SH group can be produced in several ways, notably by using SPDP to introduce PDT groups as above, and then reducing them. With PDT groups, the interchange reaction favors conjugate formation.

In conjugating whole toxins, the free -SH groups would normally be incorporated into the Ig rather than into the toxin to avoid reductive cleavage into toxin A and B chains or polymerization.[367] Introduction of free -SH groups into Ig can be achieved by reducing PDT-Ig under mild conditions with DTT without splitting interchain disulfide bridges.[334] With an average of two reactive groups per protein (-SH in one and pyridyldithio in the other) and equimolar reaction mixtures, a 20 to 40% yield of a 1:1 conjugate can be obtained by disulfide interchange coupling.[367] In conjugating a toxin A chain, the procedure would be to react PDT-Ig with the reduced single chain toxin possessing a single -SH group. With two PDT groups per Ig and a two- to threefold molar excess of A-chain-SH, the yield of a 1:1 conjugate is of the order of 50%.[367] The reaction of an S-sulfonated protein with Fab-SH is another example of coupling via an -SH group of which there is only one per molecule.[370]

N-succinimidyl iodoacetate can be reacted in neutral buffer with epsilon amino groups to introduce iodoacetyl groups into a protein.[351,367,371] Tritiated reagent may be used to monitor incorporation which can be limited to a few sites, e.g., two in the case of ricin[372] and the MAB studied by Garnett et al.[351] Analogous reagents include the anhydride and *N*-hydroxysuccinimide ester of bromo-acetyl-*p*-aminobenzoic acid.[364] Coupling of the haloacetyl derivative will occur smoothly at pH 7 under nitrogen with a second protein molecule carrying a free -SH group, e.g., produced by reduction of a PDT-substituted protein.[351] This produces a thioether bond rather than a disulfide. The yield of 1:1 conjugate is also between 20 and 40%. Whole toxin conjugates prepared with iodoacetylated toxin will retain the original disulfide linkage between the A and B chains, whereas the reaction between iodoacetylated Ig and a toxin A chain produces a thioether-linkage.

C. Purification of Conjugates

The following comments will illustrate some of the difficulties in obtaining homo-

geneous purified preparations and, more broadly, underline the desirability of designing linkage methods that facilitate production of well-defined conjugates. If a polyclonal Ig is used, it will be heterogeneous and this mixture of molecular forms will result in a corresponding mixture of conjugate species. Whatever amino acid side chain or carbohydrate residue is chosen for the linkage reaction, there will be variations in the number and distribution in different Ig molecules. This will lead to variations in the extent of agent incorporation which will be superimposed on variations arising from the linkage reaction itself as discussed below. The use of MAB avoids heterogeneity in conjugate structure arising from heterogeneity in Ig structure. However, heterogeneity can still arise with monoclonal Ig. Linkage sites, e.g., a particular amino acid side chain, will occur in different locations over the accessible surface of the Ig, and differing neighboring groups will influence the chemical reactivity at each individual site. A given site in a given molecule may or may not become substituted, resulting in a distribution in overall incorporation for a population of Ig molecules. Also, the chemical linkage reaction may not be specific and incorporation may occur at more than one class of site, with possible variation in retention of inherent agent activity. A further ramification of this heterogeneity is the fact that some incorporation may occur in a variable manner in the antigen-binding site so as to affect antigen binding. Thus, the population of conjugate molecules may vary, not only with respect to total molar incorporation per Ig molecule, but also with respect to affinity for antigen.

Differences in charge, size, hydrophobicity, etc. may not be adequate for separation of the mixture of conjugate species by conventional methods. In practice, purification has been carried out in many cases simply by gel filtration to remove unbound agent, chemical reaction side products, and grossly aggregated Ig. This technique may fail to effectively resolve the desired conjugate from polymers of Ig or from unbound high molecular weight agents such as protein toxins. However, isoelectric focusing, preparative HPLC, various gel electrophoresis techniques, etc. can achieve partial if not complete resolution. Affinity techniques based on antigen binding are in principle capable of eliminating conjugate molecules with little or no binding capacity, while those based on agent binding can eliminate Ig molecules with little or no agent incorporated.

In some cases, purification techniques with superior resolution have been used. For example, Uckun et al.[373] used protein A-Sepharose to purify a conjugate of pokeweed antiviral protein and a monoclonal IgG1. Unconjugated toxin was not adsorbed. The IgG1 bound weakly to protein A, allowing elution under mild conditions, i.e., with pH 5.5 citrate. A disadvantage of this approach is that it will not resolve free Ig and conjugate unless there is a distinct difference in the affinity for protein A. In another instance, Kato et al.[355] used preparative disc PAGE to purify drug-pGA-Ig conjugates, leading to reported purities for the ternary antibody conjugates of the order of 90%.

Purification of toxin-antibody conjugates has been outlined by Cumber et al.[367] With protein-protein conjugates, simply eliminating a low molecular weight fraction by gel filtration will not resolve various polymers — trimers, tetramers, etc. — hence the desirability of linkage methods that optimize production of 1:1 conjugates. Gel filtration using Sephadex® G-200 or Sephacryl S-300 gels will allow separation of unreacted toxin chains or whole toxins (which are weakly absorbed by the matrix) and higher polymers. Protein A absorbants will allow removal of free toxins since they retain antibodies if they are of the appropriate class. Conversely, free antibody can be removed by treating conjugates with an adsorbant with affinity for the toxin moiety.

D. Characterization of Conjugates

Characterization of conjugates includes: (1) extent of incorporation of agent in Ig; (2) extent of preservation of the inherent activity of the conjugated agent; and (3)

extent of preservation of antibody activity of the Ig. The objective for therapy is selective toxicity to a target tissue in vivo. Thus, testing of conjugates has tended to focus on measurements of growth inhibition of cells in culture or inoculated into experimental animals, without extensive prior chemical or immunological characterization. Problems in characterization can arise from the heterogeneity of conjugate preparations. However, proper characterization should aid in the design of optimally active conjugates.

1. Cytotoxic Agent Incorporation and Activity

If the agent possesses a chromophoric group, incorporation may be determined spectrophotometrically once the molar absorption coefficient has been determined. If a radioactive agent is available or can be synthesized, isotope counting can provide a sensitive method for measuring incorporation. Toxin-antibody conjugates have been characterized by nonreducing SDS-PAGE that can resolve molecular species consisting of toxin linked to Ig in molar ratios of 0:1, 1:1, 2:1, 3:1, etc.[367,368] Also, toxin incorporation can be determined by radioiodinated toxin[367] or by solid-state RIA using antitoxin antibodies.[374]

Assay of retention of agent activity may be relatively straightforward if the agent can interact with the molecular target when it is in bound form. Assays that may be applicable include enzyme inhibition, complexing with DNA, etc. The activity in conjugate form of an alkylating agent such as chlorambucil has been determined spectrophotometrically[375] and by a fluorescence method.[376] These methods can measure both the extent of incorporation and retention of alkylating activity. Advantage may be taken of direct interaction with a target molecule as in the case of MTX which binds very strongly to DHFR. The capacity of the bound antimetabolite to combine with the enzyme is determined by measuring the inhibition of enzyme activity.[345] If a decrease in activity is observed, such determinations do not distinguish between a general decrease in binding affinity for conjugated drug and a total incapacity to bind for a fraction of the conjugated drug. Binding assays using an immobilized enzyme should help in characterization but have not been applied to MTX conjugates. In the case of disulfide-linked toxin conjugates, the toxin moiety can be assayed by measuring protein synthesis with a reticulocyte lysate.[377,378] When release of a bound agent is required for cytotoxicity, then assay methodology can be designed to take this into account. Measurement of inhibition of growth of bacterial cells in culture that are sensitive to the agent has been used but cannot always be extrapolated to effects on human or experimental tumor cells.[359]

2. Antibody Activity

Numerous techniques such as indirect immunofluorescence with target cells and ELISA have been used to evaluate conjugates for retention of antibody activity and this topic is beyond the scope of the present review. Lindmo et al.[379] have recently described a convenient analysis to establish the immunologically active fraction and the affinity constant. Their procedure is based upon data from the measurement of the binding of isotopically labeled antibody to target cells. This would be useful in distinguishing the presence of a fraction of conjugate molecules that is incapable of binding to target antigen and might be eliminated from the preparation by an affinity technique.

E. Binding of Antibodies to Drug-Carrying Liposomes

The amount of a drug that an antibody can carry may be greatly increased if the antibody is bound to liposomes into which the drug is incorporated instead of being linked directly to the drug. This is because lipid vesicles can contain many more drug

molecules than can be incorporated into an Ig molecule. Drugs do not need any chemical manipulation for liposomal encapsulation and they are protected by the liposomal membrane during transit. Furthermore, the altered pharmacokinetic and metabolic properties of liposome encapsulated agents (e.g., slower clearance and catabolism) may be therapeutically advantageous.[380,381] The stability of the antibody-liposome bond is of prime importance. Several methods for coupling Ig to liposomes via a stable covalent linkage have been reported,[382-387] but the efficiency of binding of some is very low.[383,387,388] In several other methods,[386,387] dialysis in the presence of a detergent is used to bind Ig onto lipid vesicles, but the entrapped substance is likely to leak out. Martin et al.[385] and Goundalkar et al.[389] have described methods which have higher efficiency of binding Ig and which avoid dialysis in the presence of a detergent. Ghose et al. and others[381,390] have demonstrated that, at least in vitro, antibody-linked drug-carrying liposomes aggregate around and bind to target cells. However, whether the liposomal content can reach intracellular target molecules in vitro or in vivo remains to be conclusively demonstrated.[380,381] Recently, Matthay et al.[391] showed that antibody-coated small unilamellar vesicles containing MTX-gamma-aspartate aggregated around and were endocytosed by target mouse tumor cells. The size of liposomes appears to be critical since adequate endocytosis could take place only with small unilamellar vesicles. Results of studies on the tumor uptake of such targeted small unilamellar vesicles in vivo will be of interest.

V. CURRENT STATUS OF CYTOTOXIC-AGENT-ANTIBODY CONJUGATES

In this section, the authors will discuss the properties of selected conjugates of low molecular weight drugs and protein toxins that illustrate the scope of the field, emphasizing more recent reports.

A. Antibody-Bound Drugs

Drugs of greatest current interest for antibody conjugation include daunorubicin and adriamycin, MTX, chlorambucil, vindesine, and mitomycin C. Reports have also appeared on bleomycin, FUR, ARA-C, and neocarzinostatin. Other drugs that mainly were the subject of earlier studies have been reviewed elsewhere.[12,14,340] These include phenylenediamine mustard,[392] Trenimon,[292] and melfalan.[393]

1. Chlorambucil

Chlorambucil can bind noncovalently to Ig and possesses a carboxyl group which allows for application of various covalent linkage methods.[14,340] Both covalent and noncovalent antibody conjugates that retain drug and antibody activity have been studied by several groups.[128,392,394-402] Factors affecting noncovalent binding have been discussed.[403-406] Covalent conjugates using dextran have also been prepared.[392] The carbodiimide-mediated linkage gave recoveries of antibody activity that varied depending on the antibody used for conjugation. With polyclonal IgG against EL4 cells, up to 40 mol/mol were incorporated with substantial retention of anti-EL4 antibody activity.[398] In addition to ECDI-mediated linkage,[398] the isocyanate derivative of chlorambucil has been reported to react with an anti-CEA antibody to yield a conjugate with 25 mol of chlorambucil per mole of Ig and retention of alkylating activity,[376,402] but the antibody activity was not reported. When this conjugate was assayed in vitro on a human colon adenocarcinoma line with continuous exposure, the order of effectiveness was specific conjugate > chlorambucil > a mixture of drug and specific antibody > nonspecific conjugate. The antibody alone was not cytotoxic. Short-term exposure to the agent also showed that the conjugate was more potent than the free drug. The conjugate was

no more effective than the free drug on a non-CEA-producing cell line. In vivo assays were not carried out.

Using the noncovalent method of linkage,[403] between 100 and 500 mol of active chlorambucil per mole of Ig (after dialysis against PBS overnight) were bound to two affinity-purified polyclonal anti-idiotype antibodies against two IgM-secreting human lymphoblastoid cell lines (RPMI-6410 and RPMI-8392).[401] However, the extent of retention of antibody activity in the conjugate was not reported. A complement-independent ^{51}Cr release cytotoxicity assay showed that the specific conjugate was at least threefold more cytotoxic toward target cells than nontarget cells and more effective than free drug, drug plus antibody, or drug plus normal rabbit Ig.

2. MTX

MTX was one of the earliest choices for preparing drug-antibody conjugates.[12,14,340] This antimetabolite is highly potent; its complex formation with the target enzyme DHFR is virtually stoichiometric, although an excess is required intracellularly for effective action.[407] MTX contains both amino and carboxyl groups, but manipulation of the carboxyl groups is less likely to interfere with enzyme inhibition.[345] The drug is commercially available with tritium labeling, either in the glutamate or *p*-aminobenzoyl moieties so that isotope methods can be employed to determine incorporation and uptake and catabolism of its conjugates.[295,307,408] MTX was rendered selectively toxic to tumor cells when covalently coupled by an active ester method to a rabbit IgG antibody against a TAA on the surface of mouse EL4 lymphoma cells; this conjugate inhibited tumor growth more effectively in vivo than did free MTX or MTX linked to normal rabbit globulin (NRG).[345]

More recent studies have dealt with conjugates synthesized with antimelanoma antibodies and their testing in a human solid tumor xenograft model, which is more pertinent to the clinical setting than ascites tumor models. Antimelanoma MAB 225.28S reacts with a high molecular weight MAA on the surface of human melanoma cells. Using radioiodine-labeled MAB 225.28S which had been purified by ion-exchange chromatography and gel filtration, Ghose et al. have shown that intravenously injected antibody selectively localizes in xenografts of human melanoma in nude mice.[139] It was also shown that sera from melanoma-bearing nude mice did not obstruct binding of the specific MAB to melanoma cells. MTX was covalently linked to MAB 225.28S to produce a conjugate with an incorporation ratio of 5 to 6 mol/mol of IgG.[302] It gave a single band on SDS-PAGE, and there was no decrease in membrane immunofluorescence titer of antibody activity at the incorporation ratios used. Conjugated MTX was approximately half as inhibitory toward DHFR as the free drug. In human melanoma M21 xenografted nude mice, multiple i.v. injections of the MTX-MAB conjugate inhibited the tumor more effectively than the MAB, the free drug, or the drug-linked to normal mouse IgG.[302] There was also no tumor inhibition by a mixture of MTX and the MAB. A polyclonal rabbit antihuman melanoma IgG carrying equivalent amounts of MTX was tumor inhibitory but less so than the MTX-MAB conjugate. In vitro experiments showed that the MTX-MAB conjugate was less cytotoxic than free MTX, a result that has been obtained with other conjugated drugs.[292,409]

Garnett et al.[351] prepared a conjugate of MTX and an antibody against a human osteogenic sarcoma cell line, MAB 791T/36, which had been purified by chromatography on protein-A Sepharose. They used HSA as a multivalent intermediary. The methodology entailed introduction of sulfur-containing moieties preparatory to forming a thioether linkage. HSA was reacted with SPDP to yield PDT-HSA, with an incorporation ratio of 2.[354] MTX was incorporated into the PDT-HSA intermediary using ECDI at a molar ratio of 100:70:1 (ECDI:MTX:PDT-HSA). The monomer peak obtained by gel chromatography of PDT-HSA-MTX (molar incorporation

ratio 32 mol of MTX per mole of HSA) was used for synthesizing the ternary conjugate. PDT-HSA-MTX was reduced to the corresponding free -SH derivative by treatment with DTT and reacted in a 4:1 molar ratio with iodoacetyl-IgG (molar incorporation ratio 2) which had been prepared by reaction of IgG with *N*-succinimidyl iodoacetate.[371] Fractogel TSK HW55s partially resolved MTX-HSA, unconjugated IgG, the ternary conjugate, and higher polymers. ^{131}I labeling of HSA aided in characterizing column fractions and allowed calculation of the molar ratio of HSA to IgG in addition to MTX in the final product. The final stoichiometry in the pooled ternary conjugate fractions was reported to be 1 to 3 mol of MTX-HSA (each carrying 32 mol of MTX) per mole of MAB 79IT/36. A competition assay using flow cytofluorimetry showed that 28% of antibody activity was retained after conjugation.

In a cytotoxicity assay based on ^{75}Se-methionine incorporation in vitro, MTX bound to HSA was much less toxic than free MTX toward either target or nontarget cells. MTX in the ternary MAB conjugate was about as toxic as the free drug toward target cells and as toxic as MTX-HSA toward nontarget cells. Antibody alone had little effect on either type of cell. Cytotoxicity assay based on pulse exposure to the test-agent of 15 min followed by culturing in test agent-free medium showed that the ternary conjugate was more toxic than free MTX toward target cells but essentially nontoxic toward nontarget cells. Finally, a clonogenic assay with 5 days of continuous exposure gave I_{50} values of 4 ng/mℓ for both MTX and the ternary conjugate. A competition experiment with free antibody showed the cytotoxicity was strictly dependent on binding of the drug-carrying specific antibody to the cells.

Embleton et al. have evaluated the effectiveness of a MTX-HSA-MAB-791T/36 conjugate on human sarcoma xenografts in nude mice.[410] Multiple doses of test agent of 2.5 mg/kg (MTX equivalent) were given up to day 22 after s.c. tumor inoculation. The tumor diameters at day 32 were 5.6, 3.1, and 8.6 mm when the test agents were MTX, MTX-HSA-MAB, and PBS, respectively. However, six of ten mice treated with free MTX died, whereas there were no toxic deaths in the conjugate-treated group. Garnett and Baldwin have recently improved the synthesis of MTX-HSA-MAB conjugates.[410a] They omitted the SPDP-based introduction of a free -SH group in the HSA and instead reduced the MTX-HSA binary complex itself with DTT to provide the -SH site for conjugation with iodoacetylated antibody. Up to 38 mol of MTX were incorporated per mole of HSA and the ternary conjugate retained 32 to 35% of antibody activity. ^{75}Se-methionine uptake and colony inhibition assays demonstrated that the new conjugate was more toxic than MTX toward antigen-containing cell lines and was more toxic than the conjugate produced by their former method.[351] Separation of different molecular weight fractions by HPLC showed that all were cytotoxic.

Instead of HSA, Manabe et al.[360] have used a dextran-based method to conjugate MTX to IgG which is essentially the same as that used for bleomycin[358] and for mitomycin C.[359] Polyaldehyde dextran was incubated with a murine anti-HLA IgG for 24 hr followed by addition of MTX and further incubation for 24 hr giving the composite imine product. Finally, stabilization was achieved by borohydride treatment. The best MTX incorporation was approximately 9 mol/mol of IgG, not very high considering the use of the multivalent spacer. Inhibition of DHFR activity was used as a gauge of retention of drug activity. The incorporated MTX assayed by spectrophotometry retained 46% of its DHFR inhibitory activity. Membrane immunofluorescence assay showed retention of 85 to 90% of antibody activity.

Cytotoxicity assays used two bases for comparison. In one, more conjugated MTX than free MTX was added to cell suspensions to compensate for the apparent loss of DHFR inhibitory effect on conjugation. Cytotoxicity assay after 3 days of continuous exposure to the test agents revealed that the conjugate was 18-fold more toxic to target

BALL-1 cells than the free drug and 7-fold more toxic than the corresponding nonspecific Ig conjugate. All these test agents had similar toxicity toward nontarget cells. Thus, conjugation increased cytotoxicity irrespective of specificity. Also, the superiority in vitro over free MTX was in contrast to the behavior of the conjugate in which MTX was directly linked to antimelanoma antibodies studied by Ghose et al.[302]

The second set of assays entailed a 2-hr pulse exposure to equimolar concentrations of MTX, free or conjugated. The I_{50} for the specific conjugate measured against HLA-containing NALL-1 cells was 1.24×10^{-6} *M* and measured against HLA-lacking NS-1 cells was 2.9×10^{-6} *M*, the difference being statistically significant. Free MTX was equally cytotoxic toward these cell lines, having an I_{50} around 10^{-6} *M*. Binary MTX-dextran conjugates were not investigated. Thus, the cytotoxic properties can vary substantially depending on the assay conditions. After pulse exposure, the specific conjugate was not a great deal more effective against target cells than against nontarget cells. It was suggested that the HSA intermediary lends itself better than dextran[411] to development of improved lysosomotropic linkages.

The approach to conjugation of MTX taken by Manabe et al.[360] was distinctive in that it involved reaction at the pteridine ring moiety. Kulkarni et al.[345] had conjugated MTX through its carboxyl groups because of the importance of the pteridine ring in interaction with DHFR. In this study by Manabe et al.,[360] it was possible to synthesize cytotoxic conjugates using the amino groups of the pteridine ring. Moreover, the bound drug was still at least partly capable of interacting with DHFR and the authors pointed out that possibly only the MTX molecules linked via the 2-amino rather than the 4-amino group are active, the 4-substituent being the more critical one. Linkage through the latter group could contribute to loss of ability to bind to the active site of DHFR after conjugation. Cytotoxicity could be postulated to occur in the cell culture assay due to release of free drug by cellular catabolism, but this would not explain the DHFR inhibition findings with the intact conjugate.

Shen and Ryser[343] have developed a system for targeting to Fc receptor-bearing cells based on the fact that Fc receptors will bind antigen-antibody complexes. A polyclonal rabbit anti-HSA antiserum was used to form immune complexes with a binary conjugate of tritiated MTX and HSA (18 mol of drug per mole of HSA). Cytotoxicity was assayed by exposing monolayer cultured cells to test agent and then counting cells after 5 to 10 days growth. Tumor cells bearing Fc receptors (lines M5076 and WEHI) became associated with significant amounts of tritium and were killed only in the presence of both MTX-HSA (30 n*M* with respect to MTX) and antiserum, not MTX-HSA alone. That the cell-associated radioactivity represented uptake was shown by the fact that MTX-transport deficient cells as well as cells capable of taking up free MTX were inhibited from growing by MTX-HSA and antiserum. Extracellular release of MTX from the HSA could not have led to inhibition of the transport deficient cells. These studies also showed that phagocytic and nonphagocytic cell lines with Fc receptors were equally susceptible to HSA-conjugated MTX plus antiserum. Protein A, which blocks the Fc interaction with the surface receptor, counteracted cytotoxicity, as did excess unsubstituted HSA.

3. Anthracycline Glycosides

Daunorubicin has been conjugated by several methods to different macromolecular carriers.[357] Adriamycin has been less well studied and behaves differently toward certain linkage procedures. An important early comparative study by Hurwitz et al.[412] showed that the homobifunctional reagent glutaraldehyde could form conjugates of daunorubicin by linking presumably via the drug amino group and protein amino groups, but with serious aggregation problems. Single-stage coupling with a water-soluble carbodiimide resulted in substantial loss of activity. Periodate-based coupling

turned out to be the best of the three approaches tried at that time. Direct conjugation was achieved by oxidizing the sugar residue and forming imines with protein amino groups, resulting in incorporation of 2 to 5 mol of drug per mole of Ig. Stabilization was accomplished by treatment with borohydride. The chemical structure of the linkage group resulting from the periodate method has not been well worked out. It has been suggested[347,357] that oxazolidine derivatives are formed or that not all imines are reduced since daunorubicin conjugated by a nonhydrolyzable single sulfur link did not exhibit drug activity in vitro.[347] A number of other conjugation approaches applied to this drug have also been outlined in the section on methods of linkage.[333,338,339,349,353]

Several glutaraldehyde-based direct conjugation studies illustrate different approaches to targeting. Sehon and co-workers coupled daunorubicin to a goat antifibrin antibody, the rationale being that fibrin is present in both animal and human tumors and thus may be a suitable target.[413] The conjugate had 1 to 2 mol of drug per mole of IgG; antibody activity was retained. They used a ^{131}I-labeled antibody to demonstrate that localization occurred in vivo.[414] In vitro cytotoxicity assay on a methylcholanthrene-induced guinea pig sarcoma revealed the conjugate to be equally effective as free daunorubicin.[413] Multiple intratumoral injections into established sarcomas in guinea pigs led to complete tumor rejection in 50% of the animals. Neither free drug nor antibody inhibited tumor growth. While tumor inhibition after local injection could be due to sustained release of free drug at the tumor site or release intracellularly after endocytosis, these authors pointed out that tumor immunity induced by conjugate action on tumor cells could also be involved.[413]

To minimize polymerization and precipitation of Ig during aldehyde-based conjugation procedures, Belles-Isles and Page[415,416] subjected daunorubicin and polyclonal or monoclonal rabbit antihuman CEA IgG to a short incubation at 37°C with 0.01% glutaraldehyde at pH 7.2. They obtained a conjugate with an incorporation ratio of 2 mol/mol of Ig. Antibody activity was not reported. Three different in vitro assays, including continuous and pulse exposures, revealed the conjugate to be the most potent inhibitor. For in vivo assay, this group injected intraperitoneally every other day for 30 days a polyclonal anti-CEA-IgG-daunorubicin conjugate (5 mol of drug per mole of Ig) into nude mice carrying xenografts of LoVo cells measuring approximately 4 mm in diameter.[417] The conjugate, free antibody, and free drug were not significantly different from PBS controls during the first 30 days. However, growth of tumors in conjugate-treated animals then leveled off during next 40 to 50 days, before finally increasing again. Prior studies had demonstrated the localization of labeled anti-CEA antibody in human colonic carcinoma xenografts in nude mice.[418-420]

A comparative study of four different direct linkage groups has been carried out by Gallego et al.,[334] using the same mouse antihuman osteogenic sarcoma IgG2b 791R/36 used by Embleton et al.[421] to prepare MTX conjugates. Conjugates 1 and 2 were prepared from 14-bromodaunorubicin. For conjugate 1, the coupling procedure was based on that of Zunino et al.,[335] i.e., reaction of a 25-fold molar excess of 14-bromodaunorubicin with amino groups of the Ig at pH 7.5. Zunino et al.[335] had shown that protein conjugates prepared with this derivative inhibited colony formation by HeLa cells, but to a substantially lower extent than the free drug. For synthesis of conjugate 2, SPDP was first used to introduce three to four -SH groups into the IgG; reaction at pH 4.5 with a tenfold molar excess of bromodaunorubicin produced a thioether-linked conjugate. The bromo derivative was reported not to react with protein amino groups under these conditions.[334] Another linkage method entailed incorporation of an acid labile *cis*-aconityl spacer linked to both daunorubicin and Ig by amide bonds to form conjugate 3. Coupling at pH 7 was carried out by adding ECDI to IgG mixed with a 25-fold molar excess of carboxy drug. Conjugate 4 was similarly prepared, except that the spacer was a succinyl group which should not be as readily

susceptible to hydrolysis in the lysosomal milieu.[333] The incorporation was 3 to 4 mol of drug per mole of IgG for all daunorubicins. Three assays were used to measure retention of antibody activity: direct and indirect membrane immunofluorescence and competitive inhibition of binding of fluorescin isothiocyanate (FITC)-labeled antibody by conjugate. Conjugate 1 showed complete retention of antibody activity in all assay systems. Conjugates 2 and 3 showed some loss of antibody activity, the extent varying with the assay method. In vitro cytotoxicity assay based on ^{75}Se-methionine incorporation, either with 24-hr continuous exposure or after pulse exposure for 30 min, showed that the succinyl-linked conjugate had no cytotoxicity. After continuous exposure, the other three conjugates were approximately tenfold less potent than the free drug and could not discriminate between target and nontarget cells. Some target cell selectivity could be seen after 30 min pulse exposures to all three conjugates. Based on retention of antibody activity, cytotoxicity, and selectivity, the *cis*-aconityl conjugate was thought to be the most effective.

The intermediary, pGA, has been used to link daunorubicin to an affinity-purified polyclonal anti-AFP antibody.[353,355] The conjugation method was as outlined under Section IV, Methods of Linkage. ECDI was used for coupling of the drug to PDT-pGA (average molecular mass 13 to 15 kdaltons) using a molar ratio of ECDI to daunorubicin to R-S-S-pGA equal to 85:13:1. In the subsequent reaction to form the ternary daunorubicin-pGA-Ig conjugate, daunorubicin-substituted R-S-S-pGA was reacted with maleimide-substituted anti-AFP Ig or with an equivalent amount of maleimide-substituted normal horse Ig (nIg) for the control conjugate. The incorporation was 10 to 20 mol of drug per mole of Ig, depending on the molar ratio (4 to 10) of daunorubicin-substituted R-S-S-pGA-to-maleimide-substituted Ig in the reaction mixture. The extent of retention of antibody activity was not reported. A cytotoxicity assay in vitro after continuous exposure of AH66 hepatoma cells to agents for 18 hr showed that the order of effectiveness was nIg < anti-AFP < daunorubicin, daunorubicin-pGA, daunorubicin-pGA-nIg < daunorubicin-pGA-anti-AFP. Normal Ig at a concentration equal to that used for the highest level of conjugate had no effect, whereas 30 μg/mℓ of drug bound in the specific ternary conjugate gave virtually total inhibition of growth. In an in vitro-in vivo assay, AH66 ascites tumor cells were exposed to the test agents in vitro at 37°C for 30 min and then inoculated intraperitoneally into syngeneic rats. The longest survival of tumor-inoculated rats was observed when the cells had been exposed to the specific ternary conjugate, namely, 53 days compared to 18 days for recipients of PBS-control cells. The survival of rats that received AFP- or daunorubicin-treated cells was also prolonged to 35 and 45 days, respectively. There was no synergism with a mixture of anti-AFP Ig and free daunorubicin. The nonspecific binary and ternary conjugates were about as effective tumor inhibitors as the free drug. When tumor-inoculated animals were given multiple doses of test agents over a 9- or 15-day period, the specific ternary conjugate gave the greatest prolongation of survival, including a proportion of long-term survivors.

The cytotoxicity observed with this ternary IgG conjugate, in which daunorubicin was linked to pGA by using ECDI, is in contrast to the observed loss of drug activity after ECDI-mediated direct linkage to an antibody.[412] It is possible that the cytotoxicity of the ternary conjugate is due to susceptibility to hydrolysis of the amide bond involving the gamma carboxyl of glutamate, releasing free drug, or that pGA can be hydrolyzed intracellularly to produce a cytotoxic fragment comprising a gamma-glutamyl derivative of the drug. It is also pertinent to determine whether the intact ternary conjugate can interact with DNA in vitro. Tsukada et al.[353] pointed out that the approach taken by Shen and Ryser[333,422,423] in comparing cytotoxicity and uptake of poly-L- and poly-D-lysine conjugates could be applicable to elucidating the mode of action of this pGA-based conjugate.

Daunorubicin was also coupled to a 12-kdalton pGA derivative synthesized by polymerization with cystamine.[355] ECDI incorporated 6.5 mol of drug per mole of R-S-S-CH_2-CH_2-pGA. Ternary drug-pGA-antibody conjugates were again prepared with maleimide-substituted anti-AFP and with nIg for the control conjugate. Preparative disc PAGE was used to achieve a reported purification of approximately 90%. The incorporation ratio of drug to Ig was 7 to 7.5 and almost all antibody activity was retained as determined by a precipitation assay with ^{125}I-labeled AFP. In vitro cytotoxicity assay after continuous exposure of AH66 cells for 48 hr to the test agents showed the order of effectiveness to be specific ternary daunorubicin-pGA-anti-AFP > daunorubicin = daunorubicin-pGA = daunorubicin plus anti-AFP IgG = daunorubicin-pGA plus anti-AFP IgG > anti-AFP = daunorubicin-pGA-nIg. There was no effect with nIg. In contrast, with a non-anti-AFP-secreting cell line, AH272, the specific and nonspecific ternary conjugates were equally cytotoxic. However, this effect was slightly less than that of the free drug under these conditions. Neither nIg nor anti-AFP Ig had any effect on this cell line.

Dextran has also been used as an intermediary to increase daunorubicin incorporation. The methodology was an extension of that used by Bernstein et al.[424] to prepare binary daunorubicin-dextran conjugates. Polyaldehyde-dextran of a molecular mass of 10 kdaltons was incubated with daunorubicin for 20 hr and then affinity-purified anti-Yac IgG was added for a further 20 hr, followed by borohydride.[425] The drug incorporation was 2 to 3 mol/mol of dextran and 25 mol/mol of IgG. There was a 40% loss of antibody activity in the conjugate. An early ternary conjugate was less effective than the free drug in vitro and was no better than the binary dextran or ternary nonspecific Ig conjugate in vivo at high doses. Subsequently produced ternary conjugates utilizing both whole Ig and F(ab')$_2$ from an affinity-purified polyclonal horse anti-rat AFP IgG[411] had a substantial loss of antibody activity, but in an in vitro assay involving continuous exposure of AH66 cells to test agents for 48 hr, the specific conjugate was claimed to be 100 times more cytotoxic than a mixture of antibody and drug unlinked, which showed modest synergism. The nonspecific conjugate was no more effective than the free drug. Preincubation of cells with the specific conjugate prior to inoculation into rats led to five out of ten long-term tumor-resistant survivors, whereas all control animals died. The i.p. administration of the specific conjugate to tumor-bearing rats gave an average survival of 64 days compared to 16 days for control animals. Average survival times ranged from 33 to 45 days for rats given free drug, specific antibody, or a mixture of the two. It was pointed out by the authors that low levels of AFP in the group receiving the conjugate showed that AFP-producing tumor cells were being selectively inhibited.

Using dextran, Hurwitz et al.[426] could incorporate 50 mol of daunorubicin per mole of an affinity-purified anti-idiotypic antibody or its F(ab')$_2$ fragment directed against a clonally expressed cell-surface IgM of a B-cell lymphoma. The ternary antibody conjugate exhibited an approximately 50% loss of binding to target cells. In vitro cytotoxicity assay based on inhibition of tritiated thymidine uptake by 38C cells showed the order of effectiveness to be free drug > specific F(ab')$_2$ conjugate > specific whole Ig conjugate = nonspecific Ig conjugate. In i.p. tumor-bearing animals, the specific conjugate was more effective than the free drug when given intraperitoneally but not much better than the free drug when given intravenously. There was a reduction in systemic toxicity of conjugated daunorubicin so that higher doses could be given, producing effective tumor inhibition. At these higher dose levels, the specific conjugate was more effective than the nonspecific conjugate, producing cures in a proportion of animals. F(ab')$_2$ conjugates were less effective than whole Ig conjugates, and increasing the tumor burden above 10,000 cells also lowered the effectiveness of conjugates.

Pimm et al.[427] conjugated 18 to 28 mol of adriamycin to a monoclonal IgG2b against

rat mammary carcinoma Sp4 following the method described for linking daunorubicin to anti-Yac IgG. An in vitro-in vivo assay, in which test agent-pretreated Sp4 cells were inoculated into rats, showed that tumor growth was retarded by the specific conjugate but not by free adriamycin or a nonspecific conjugate. When s.c. tumor-inoculated rats were treated with multiple i.p. doses of test agent, dosages of the order of 500 μg/kg for the free drug were required to inhibit tumor growth significantly, but the specific conjugate exerted a distinct effect at adriamycin doses of 30 to 75 μg/kg. Survival ranged from 30 to 50 days for control animals and from 60 to 90 days for animals treated with the specific conjugate. Antibody alone had no effect on tumor growth and synergism between free drug and antibody was not observed. These authors concluded that this conjugate and test system gave results superior to those obtained by Hurwitz et al. with anti-Yac antibodies.[425] There was evidence for preferential tumor localization of radiolabeled antibody.

Ghose et al.[13,264] successively reacted polyaldehyde dextran T40 with adriamycin and antimouse renal cell carcinoma IgG without borohydride reduction. There was no loss of drug from the conjugate on repeated dialysis. Adriamycin-dextran-IgG conjugates had less systemic toxicity than the free drug. When renal cell-carcinoma-bearing mice were given i.v. injections of equitoxic doses of test agents for 7 successive days, the order of antitumor effect was specific conjugate > nonspecific conjugate > free drug. Only the specific conjugate could produce long-term survival in a proportion of treated mice.

4. Vindesine (Desacetylvinblastine Amide)

Vindesine has been investigated for antibody-mediated targeting by Rowland and co-workers.[428-432] Rowland has pointed out that effective clinical dosages of vindesine are 10 to 100 times lower than other drugs that have been linked to antibodies, e.g., the potency is intermediate between other anticancer drugs and toxins.[428] Vindesine also has a phase-specific antimitotic effect, whereas toxins linked to antibodies could affect both dividing and nondividing cells that took up conjugate nonspecifically.[428,431] Vinblastine derivatives have a carboxy function (C23) which can be variously substituted without loss of cytotoxicity.[433] For example, desacetylvinblastine possesses a methyl ester while vindesine has an amide. The most common conjugation procedure has been to first react desacetylvinblastine with nitrous acid.[434] The resulting azide need not be isolated, but can be reacted directly with the Ig at pH 9.[428,433] The site of linkage has not been identified but is presumed to be the epsilon amino group of lysine.[433] Incorporation ratios of 4 to 11 mol/mol of IgG have been reported.[429,432] Conjugates prepared with mouse monoclonal antiosteogenic sarcoma IgG2b were shown to retain essentially full antibody activity based on a competitive binding assay.[431] Conjugates prepared with a polyclonal sheep anti-CEA preparation were observed to localize on target cells.[430]

Vindesine-antibody conjugates have been evaluated by an in vitro indirect targeting assay using tumor cells coated with an appropriate rabbit antibody.[428] The vindesine-antirabbit-Ig conjugate produced significant inhibition of antibody-coated cells but not uncoated cells. In another in vitro assay involving inhibition of incorporation of ^{75}Se-methionine into osteogenic sarcoma cells, target-specific inhibition could not be demonstrated. However, after pulse exposure, the conjugated drug was less cytotoxic by several orders of magnitude than the free drug but selective toward target cells.[429,431] When vindesine was conjugated to a polyclonal sheep anti-CEA antibody, exposure of CEA-bearing CALU-6 cells showed that the conjugate was considerably more potent than free vindesine, a mixture of vindesine and antibody, or a conjugate synthesized with normal sheep IgG.[428] Evidence for localization of the specific conjugates in sarcoma-bearing nude mice was obtained by using a radioiodinated conjugate. The tissue-

to-blood ratio was of the order of threefold greater for tumor tissue than for any other tissue. In other experiments, it was shown that the cytotoxicity of vindesine conjugated to a monoclonal anti-p97 antibody toward different melanoma cell lines varied directly with the surface density of p97 antigen. Repeated injections (ten during 34 days) of two different conjugates with monoclonal anti-CEA antibodies (an IgG2a and an IgG1, respectively) were administered to human colorectal carcinoma-bearing nude mice. There was substantial suppression of tumor growth compared to controls up to approximately 60 days with both conjugates, after which tumor growth accelerated in the animals given the IgG2a conjugate. In contrast, the IgG1 conjugate suppressed tumor growth until 90 days, the reported duration of the experiment.[429] Ford et al.[432] were able to show that a radiolabeled conjugate of vindesine and a polyclonal sheep anti-CEA antibody localized in five out of eight patients with advanced colorectal and ovarian carcinoma. No measurements of antitumor effects were reported.

5. Nucleosides

Hurwitz et al.[435] have investigated two nucleotide analogs, ARA-C and FUR, which are competitive inhibitors of DNA synthetic enyzmes. Both possess vicinal hydroxyl groups in their sugar moieties, and cytosine has an amino group as a potential linkage site. Modification of either of these groups might be expected to affect the interaction with target molecules in vivo but the amino group may be more critical. Linkage of ARA-C to an affinity-purified goat antibody against surface IgM on 38C leukemia cells was carried out by reacting polyaldehyde dextran with the drug and then with the IgM. Finally, sodium cyanoborohydride was used to stabilize the linkage. A total of 25 to 60 mol of tritium-labeled drug was bound per mole of IgM with almost full retention of antibody activity. The antitumor effect was assayed in vitro by enumeration of viable 38C cells or by inhibition of incorporation of [methyl-^{3}H]-thymidine and/or -uridine after pulse exposure to test agent for either 2 or 24 hr. It was found that the drug-dextran conjugate and drug-dextran-antibody conjugate were somewhat more effective than the free drug at certain concentration levels. Some specificity toward target tumor cells could also be seen with the antibody conjugate.

The linkage methodology for conjugation of FUR also used dextran as intermediary, but in this case the nucleoside sugar residue was oxidized with periodate rather than the dextran. This produced two vicinal aldehyde groups capable of reacting with the hydrazide derivative of dextran. The binary hydrazone conjugate was linked to the IgM using glutaraldehyde and stabilization by reduction was not carried out. There was incorporation of 7 to 24 mol of tritium-labeled FUR per mole of Ig with retention of most of the antibody activity, although less than in the ARA-C conjugate. Using the same in vitro assays as with ARA-C conjugates, it was found that at high drug concentrations (1 μg/mℓ) there was no marked difference in the inhibitory effects of the free drug and the binary and ternary conjugates. The inhibitory effects of conjugates were compared to fluorouracil rather than FUR since the sugar ring in effect had been destroyed by oxidation. Thus, one could postulate that the base rather than the nucleoside would be released from the conjugate intracellularly and converted both to FUMP, which is incorporated into RNA, and to FUdMP, which inhibits thymidylate synthetase. However, these authors pointed out that it is possible that the conjugates could operate via a mechanism different from that of the free drug.

6. Bleomycin

Bleomycin has been linked to a murine anti-HLA IgG1 MAB by Manabe et al.[358] Polyaldehyde dextran was first incubated with bleomycin and then with the IgG followed by borohydride treatment. A total of 58 mol of drug was incorporated per mole of IgG. Assay of the activity of the agent by growth inhibition of *Mycobacterium*

nostatin linked to normal rabbit IgG. Luders and co-workers used SPDP to effect a disulfide linkage between the apoprotein of neocarzinostatin and an IgG1 MAB against a high molecular weight antigen (A-1-43) on the human melanoma cell line A-375.[438a]

smegmatis gave an apparent retention of 18% of drug activity. There was complete retention of antibody activity when measured by membrane immunofluorescence. The ternary bleomycin conjugate was 15-fold more toxic to cells possessing HLA than was free bleomycin but significantly less toxic than free bleomycin toward cells lacking target HLA.

7. Mitomycin C

Mitomycin C has been studied by two groups of investigators who took distinctly different approaches to conjugation.[336,359] Kato et al.[336] coupled this agent to a polyclonal horse anti-AFP Ig at the N-1a position by using a glutaric acid-based spacer arm. A 4-carboxybutyryl substituent was attached to the mitomycin C by reaction with glutaric anhydride. The corresponding active ester was then synthesized by reaction with NHS and dicyclohexylcarbodiimide. Coupling with the Ig was effected with a 20-fold molar excess of the drug derivative to achieve an incorporation ratio of 8 mol of drug per mole of Ig. This method avoids Ig polymerization and allowed higher protein recoveries and higher incorporation ratios than a previously used cyanogen bromide method.[436] The aziridyl amide linkage was slowly hydrolyzed with a half-life of 2.6 days at pH 7.4 and 0.4 days at pH 4, which may facilitate lysosomal breakdown. A fivefold increase in cytotoxicity in vitro toward target AH66 hepatoma cells was achieved with the specific conjugate in comparison with a mixture of free drug and antibody. A nonspecific conjugate was approximately as inhibitory as the free drug. Multiple i.p. injections over a 10-day period of the specific conjugate to i.p. tumor-inoculated rats were able to prolong mean survival from 17 days (saline controls) to 57 days. Mean survival was 23 days for the free drug group and 28 days for the nonspecific conjugate group. In the second approach, Manabe et al.[359] used dextran to prepare a conjugate with monoclonal anti-H-1 IgG1 antibody in essentially the same way as described for MTX,[360] etc. except that the reaction order was Ig followed by mitomycin C. The incorporation was 88 mol of drug per mole of IgG. Based on an assay of growth inhibition of *Escherichia coli*, conjugated mitomycin C was found to be 2.4% as effective as the free antibiotic. Membrane immunofluorescence of target tumor cells revealed substantial retention of activity in the conjugates and the conjugate was tenfold more cytotoxic toward target cells in vitro than the free agent.

8. Neocarzinostatin

Neocarzinostatin consists of an acidic protein complexed to a cytotoxic chromophore producing DNA damage. Kimura et al.[437,438] have conjugated this antibiotic protein to a rabbit IgG against a human leukemia cell line (NALL-1) using ECDI. Indirect membrane immunofluorescence (FITC-labeled goat antirabbit IgG) showed that antibody activity was retained. The specific conjugate inhibited growth of NALL-1 cells to the same extent as free neocarzinostatin in a continuous exposure assay but, after pulse exposure, to a greater extent than neocarzinostatin alone or neocarzinostatin conjugated to normal rabbit IgG. Inhibition of tritiated thymidine deoxyriboside uptake by antibody-linked neocarzinostatin was greater than by free neocarzinostatin or antibody or by neocarzinostatin plus antibody unlinked. The order of effectiveness after i.p. administration of agents to immunosuppressed Syrian hamsters inoculated intraperitoneally with 5×10^6 BALL-1 cells was specific conjugate > neocarzinostatin linked to NRG or neocarzinostatin plus antibody or neocarzinostatin > antibody or saline. If tumors were inoculated subcutaneously, the effectiveness of the specific conjugate was no greater than neocarzinostatin alone, neocarzinostatin plus antibody, or neocarzinostatin linked to normal rabbit IgG. Luders and co-workers used SPDP to effect a disulfide linkage between the apoprotein of neocarzinostatin and an IgG1 MAB against a high molecular weight antigen (A-1-43) on the human melanoma cell line A-375.[438a]

The ternary conjugate was purified with Protein A Sepharose and then the biologically active chromophore of neocarzinostatin was added in excess to form the specific drug complex. In this method, the apoenzyme is essentially being used as an intermediary. The specific conjugate was 100-fold more toxic toward A-375 cells, as measured by tritiated-thymidine uptake, than free neocarzinostatin or neocarzinostatin conjugated to normal mouse IgG1. There was also a selectivity factor of 40 to 50 observed in comparing the action of the specific conjugate on antigen-positive and antigen-negative cells.

B. Antibody-Bound Toxins

Considerable interest has developed in the use of catalytically active protein toxins for conjugate preparation. In the category of catalytic agents, one could include essentially any enzyme capable of disrupting membrane constituents (e.g., phospholipase-C^{14}), intracellular DNA or RNA (e.g., ribonuclease), or various intracellular proteins. However, the emphasis has been on the group of bacteria- and plant-derived polypeptide/protein agents categorized as toxins. Toxins that have been conjugated to antibodies include gelonin, pokeweed antiviral protein, abrin, ricin, diphtheria toxin, and *Pseudomonas* exotoxin. There are two main types: single-chain toxins and linked polypeptides. The linked polypeptides consist of a A chain which is the cytotoxic entity and a B chain which is a lectin responsible for binding to cell surface carbohydrate moieties. For example, the B chain of ricin binds at cell surface galactose-containing moieties and also plays a role in transfer of the A chain to the cytoplasm. If the B chain is removed by cleavage of the disulfide linkage, then the isolated A chain is likely to exhibit cytotoxicity toward whole cells that is of the order of 5 to 6 logs less than the intact toxin.[439,440] However, the isolated A chain retains its ability to inhibit protein synthesis as measured in a cell-free system. The literature has been reviewed in recent years.[14,18,29,340,344,362,364-366,440-443] Also, the authors have considered some of the special features of linking a polypeptide or protein to an antibody in Section IV on methods of linkage.

In general, there are two major modes of action by which agents exert their cytotoxic effect. An agent can either interact stoichiometrically with its target molecule and block the target molecule's normal function or it can enzymatically modify the target molecule so that it is rendered nonfunctional. If action is stoichiometric, then a sufficient concentration of the agent must be attained to incapacitate a critical proportion of the target molecules. Incapacitation will depend on the relative concentrations of agent and target molecule and the equilibrium constant governing the interaction between them. The interaction between MTX and DHFR is illustrative. MTX binds extremely tightly to this enzyme and thus inactivates it. However, the usual intracellular level of DHFR apparently exceeds that required for cell viability by a substantial margin so the required extent of inactivation is high. It has been shown that the intracellular concentration of MTX must exceed that of DHFR to be cytotoxic.[407] Furthermore, the fraction inactivated may drop below the critical value during therapy because of increases in the intracellular level of DHFR or decreases in its affinity for MTX.

Agents that exert their effect by catalyzing an inactivating reaction can be cytotoxic at very low concentrations. They can in principle continue to act on newly synthesized target molecules as long as they themselves are not inactivated, e.g., by intracellular hydrolases. It has been estimated for at least certain toxins that as little as one molecule per cell may be sufficient for exerting a cytotoxic effect.[444,445] This very high potency renders the requirement for specificity in the carrier even more stringent and requires that the purification procedure applied to a conjugate eliminate, to the greatest extent possible, residual contamination by unreacted whole toxin molecules. In the case of antibodies, this implies low cross-reactivity toward antigens on normal cells, low levels

of the tumor antigen marker in normal tissues, and low uptake by phagocytic cells. Since the targets of protein toxins are intracellular, endocytosis of conjugated toxin or its A chain is essential. As with agents in general, an ineffective conjugate will result if the carrier antibody has low affinity or the complex with the target antigen is not easily endocytosed because of size or other factors relating to the nature of the antibody or its target antigens. Theoretically, for specific targeting, it is tempting to remove the toxin B chains that bind to a wide variety of cells so that the specificity of the carrier antibody selectively targets the A chain. However, there is a wide variation in the uptake of A chain conjugates. The B chain seems to augment the internalization of conjugates. For example, it has been observed that the presence of the ricin B chain in conjugates seems to facilitate action of the A chain on target cells even when direct B-chain binding to cell membrane receptors is blocked by lactose.[446-448] Nevertheless, active A-chain conjugates have been obtained.

For designing effective whole toxin conjugates, it is desirable to avoid intra- and intermolecular cross-linkage that can result in loss of antibody and toxin activities, e.g., through blockage of the respective combining or active sites, through covalent linkage between the A and B chains of the toxin, etc.[449] A common choice of linkage group is the disulfide bridge, and its utility for this purpose is illustrated by comparative studies with the linked polypeptide toxin, abrin, conjugated to antimouse lymphocyte IgG by two different methods.[449] SPDP was used to produce a disulfide bridge conjugation and the NHS ester of chlorambucil to produce an amide and pipirazine ring conjugation.[364] The incorporation ratio in this application was close to one. Both these reagents have been described in considering methods of linkage. Abrin conjugated by either method was an equally effective inhibitor of protein synthesis in a cell-free system. However, spleen cells in culture were twice as susceptible to the disulfide-linked conjugate, suggesting relatively easier intracellular release of an active A-chain moiety itself through disulfide interchange. Abrin linked by a pipirazine group involving sites on the A chain of the toxin would presumably be more stable.[449]

The role of the specific carrier in antibody-mediated targeting of toxins is illustrated by the studies of Ross et al. with diphtheria toxin.[450] Daudi cells are relatively insensitive to this toxin since 1 $\mu g/m\ell$ does not inhibit protein synthesis in cells in vitro. Anti-lymphocyte IgG-whole diphtheria toxin conjugates were prepared by a chlorambucil-mixed anhydride procedure. This conjugate was shown to be distinctly cytotoxic toward the Daudi cells; a concentration of 0.5 $ng/m\ell$ inhibited tritiated leucine uptake by 50%. The same behavior was observed when the $F(ab')_2$ fragment of this antibody was used for conjugation. Pretreatment of cells with free antibody or with diphtheria antitoxin lowered inhibition and a conjugate prepared with a nonspecific IgG was ineffective. These findings could not be duplicated with mouse spleen cells and a corresponding conjugate with antimouse lymphocyte antibodies. More recently, Pirker et al.[8] have demonstrated that the in vitro toxicity of an antitransferrin receptor MAB-*Pseudomonas* exotoxin conjugate to several human ovarian cancer cell lines was directly related to the extent of binding and internalization of the conjugate. Verpamil enhanced the toxicity of this conjugate.

Antibody conjugates synthesized with whole toxins suffer from the disadvantage that the B chain in the conjugate may still promote uptake by nontarget cells, resulting in high host toxicity. In one approach to minimize nonspecific binding of the B chain, intact ricin was coupled via iodoacetylated whole toxin which produces thioether-linked conjugates characterized by lowered galactose recognition.[367] (This lack of cytotoxicity to nontarget cells does not apply to iodoacetylated whole ricin itself.) B-chain-recognition-based uptake by nontarget cells can also be blocked in vitro by high concentrations of galactose or lactose in the incubation medium to saturate binding sites for nonantibody-mediated uptake of abrin or ricin conjugates. However, a further

difficulty can arise from the fact that mannose-containing oligosaccharide substituents on antibody-conjugated toxins can promote uptake by phagocytic cells via mannose receptors.[451]

Other approaches for eliminating nonspecific binding via B chains, especially in vivo, include removal of the B chain prior to conjugation or the use of single-chain toxins (so-called hemitoxins). A useful attribute of single-chain toxins from the point of view of laboratory manipulation is that they are not toxic until conjugated to a carrier that promotes endocytosis. An example is saporin, a single-chain toxin from *Saponaria officinalis* which has been conjugated, by using SPDP, to an anti-Thy 1.1 MAB (OX7) and its $F(ab)_2$ fragment.[451a] The purified conjugate fraction used for biological testing consisted chiefly of 1:1 and 1:2 (toxin to MAB) species. The conjugated toxin did not inhibit protein synthesis in a reticulocyte lysate, indicating that the toxin must be released from the carrier protein for manifestation of cytotoxicity. The specific whole IgG or $F(ab)_2$ conjugates were strongly inhibitory toward pulse-exposed Thy 1.1-positive cells in culture (but not toward Thy 1.1-negative cells). I_{50} values for inhibition of tritiated leucine uptake ranged from 3×10^{-12} *M* to 1×10^{-10} *M*. The pattern of response to Concanavalin A (Con A) and *E. coli* lipopolysaccharide mitogens by spleen cells exposed to the saporin-MAB conjugate suggested that T-lymphocytes (which express Thy 1.1 antigen) were selectively killed. Measurement of acute toxicity toward mice indicated that conjugation of saporin to IgG increased toxicity by a factor of 8 to 16. Mice inoculated intraperitoneally with 10^6 AKR-A tumor cells and then given one i.v. injection of the specific conjugate after 24 hr, survived an average of 30 days longer than untreated control mice. Three out of eight animals remained free of ascites tumor during the experiment. (Two of these animals developed solid s.c. tumors at the site of i.p. inoculation, suggestive of relative inaccessibility.) The specific $F(ab)_2$ conjugate was substantially less effective than the IgG conjugate. Antibody alone, toxin alone, antibody plus toxin unlinked, and toxin linked to an irrelevant antibody did not prolong survival. Calibration experiments showed that the pattern of survival of treated mice given 10^6 tumor cells was similar to that of untreated mice given 10 tumor cells. This finding was interpreted to indicate 99.999% elimination of tumor cells after inoculation. Tumor cells isolated from ascites tumors that grew in conjugate-treated animals had the same sensitivity in vitro to the specific conjugate and so did not represent a resistant subclone. The corresponding anti-Thy 1.1 MAB conjugate of ricin A chain had cytotoxicity in vitro that was comparable to that of the specific saporin conjugate, but the former produced survival in vivo for tumor-inoculated mice that was equivalent to only 99% killing of tumor cells. These authors suggested that the carbohydrate moiety present on ricin A chain but not saporin may lead to clearance by the reticuloendothelial system. The linkage in the conjugate of positively charged saporin may also be more stable during transit in vivo than that in the conjugate of uncharged ricin.

Another example of a single-chain toxin is pokeweed antiviral protein. Conjugation to antibodies is reported to confer on it cytotoxicity toward eukaryotic systems that is of the same order as ricin.[452] Like ricin, the mode of action involves interference with the function of protein synthesis elongation factors.[452] Recent studies show the potential usefulness of toxin conjugates in selective elimination of human cancer cells. Uckun et al.[373,453] linked pokeweed antiviral protein to an IgG_1 MAB against human B- and pre-B-cells by a disulfide interchange method. The ratio of toxin to IgG was 2:1 as shown by RIA for the toxin polypeptide. This conjugate at a toxin concentration of 5 $\mu g/ml$ produced 80% inhibition of tritiated leucine uptake by target B-ALL cells but did not affect nontarget normal bone marrow cells. A nonspecific conjugate was without effect on the B-ALL cells. A clonogenic assay showed that almost 6 logs of killing of target lymphoma cells could be achieved in the presence of chloroquine, which potentiated cytotoxicity. Moreover, this effect was achieved under conditions where there

was a 100-fold excess of normal marrow cells. There was less than a 50% loss of pluripotent stem cells.

Most conjugates containing cleaved A chains have been synthesized by disulfide linkage utilizing the A chain -SH formed by reductive splitting of the original disulfide bridge between the A and B chains of the parent toxin, a procedure which is generally capable of substantially retaining antibody activity. This has been discussed in dealing with methods of linkage. In effect, the method preserves the original linkage group but without preserving the ancillary role(s) of the B chain in toxin transport and action as stated above. Experiments involving reductive cleavage of conjugates and isolation of released A chains allowed the demonstration that the released A chains were fully inhibitory in a cell-free protein synthesis system if the original conjugation antibody was of the IgG class.[366] However, not all chains were fully active if the original conjugation antibody was of the IgM class. An interesting comparison of linkage groups is that between disulfide and thioether bridges. One way of linking via a thioether bridge is with 6-maleimidocaproic acid to introduce a 9-atom spacer. Ricin A chain linked in this way had 30% activity in a cell-free system, and the greater stability of the thioether bond indicates that this activity is indeed due to the bound A chain and appears to reflect some steric hindrance.[366] These thioether-linked conjugates were only 1% as active as disulfide conjugates on intact cells, indicative of a failure of transport. This suggests that conjugate action involves the splitting of the toxin chain. However, introducing a longer disulfide-containing spacer, which hypothetically might be split more readily, did not improve cytotoxicity against intact cells, although activity in a cell-free system was greater.[366]

Conjugates of ricin A chain have also been prepared using the biotin-avidin system outlined under Section IV, Methods of Linkage.[342] Ricin A chain was coupled to PDT-avidin by disulfide interchange and biotin to Ig by an active ester method. The ricin incorporation assayed by inhibition of protein synthesis using a rabbit reticulocyte lysate was a little over 1 mol/mol of avidin. When BALBc spleen cells were exposed to biotinylated-antimouse Ig followed by ricin-A-chain-substituted avidin, the subsequent lipopolysaccharide response was poor and the Con A response was good. When the first exposure was to biotinylated-anti-Thy-1.2, the converse responses were observed. These results indicated specific cytotoxicity toward target cells. Treatment of cells with either binary conjugate alone did not affect responsiveness. These investigators also used the $F(ab')_2$ fragment of a polyclonal IgG against the putative T-cell replacing-factor (TRF) acceptor sites to prepare a conjugate that was capable of selectively eliminating a B-cell subset having these acceptor sites. Advantages of the sequential exposure approach were stated to be the convenience of having available supplies of toxin-substituted avidin and the potential for binding multiple ricin A chains to Ig that is substituted with multiple biotin molecules. If the sequential use of biotin-Ig and ricin-avidin were tried in vivo, the possibility arises that ricin-avidin could combine with biotin of the host, thus eliminating targeting specificity.

Another related system that might allow for in vivo targeting is that consisting of the ribonuclease-S peptide and ribonuclease-S protein. The affinity constant governing this interaction is not as large as the constant for biotin-avidin, but it is still in the range of antigen-antibody constants, i.e., 10×10^9 [454] and the coupling chemistry should not be difficult, particularly since ribonuclease is a small, stable protein. (Either of these noncovalent-binding systems ought to be feasible for preparing ternary conjugates intended for administration as such.)

Conjugates that are prepared by the linkage of the cleaved A chain of protein toxins that possess both A and B chains are generally less toxic than whole toxin conjugates by a couple of orders of magnitude. Raso et al.[455] used hybrid antibodies with one specificity for target cells and the other for ricin A chain to deliver either whole toxin

or A chain to Daudi cells. The I_{50} of the cleaved ricin A chain conjugate was 10- to 100-fold greater than that of the whole toxin conjugates, which suggests augmentation of toxicity by the B chain. Furthermore, conjugates containing cleaved A chains exhibit variable cytotoxicity toward target cells in vitro, high in the case of A chains of several toxins conjugated to antibodies directed against the transferrin receptor,[368] low in the case of diphtheria toxin or ricin A chain conjugated to anti-Thy 1.1. or W 3/25 IgG.[364] The variable properties of A chain immunotoxins are further illustrated by experiments comparing diphtheria toxin A chain and ricin A chain conjugated by a disulfide linkage to epidermal growth factor which showed that the ricin conjugate was cytotoxic toward 3T3 cells at 0.01 to 1.0 n*M*, whereas the diphtheria toxin conjugate was not cytotoxic at concentrations of the order of 30 n*M*.[7] Diphtheria toxin A chain conjugated to human placental lactogen by use of methyl-5-bromovalerimidate was also inactive against mammary gland explants, although it retained ADP-ribosyltransferase activity and the conjugate could bind to lactogenic receptors.[5,456] Nevertheless, some of the A chain conjugates have been found to be effectively tumor inhibitory in vitro as well as in tumor-bearing animals. For example, a disulfide-linked conjugate between diphtheria toxin A chain and a monoclonal IgG1 against the guinea pig L10 hepatocarcinoma has been prepared by Bernhard et al.[457] The purification procedure took advantage of the binding affinity between NAD^+ and diphtheria toxin A chain. The reaction mixture was passed through an NAD-Sepharose column which adsorbed the IgG-linked toxin and eliminated unreacted IgG. Subsequent passage through a Sephadex® G-200 column eliminated unreacted A chain. The purified conjugate bound to L10 cells and not L1 cells in vitro. L10 cells in culture were reported to be 100% killed by a 24-hr pulse with conjugated A chain at 100 n*M*, whereas antibody alone was not cytotoxic. Prior incubation with antibody alone at 1 μM completely prevented cell killing by the conjugate. The anti-L10 cell IgG1 was shown to localize in tumors in vivo,[458] so the antitumor effect in vivo was assessed by treating intradermally tumor-inoculated guinea pigs with conjugate intravenously on day 2 or on day 7 after inoculation. Tumor growth was slightly inhibited by the treatment on day 2 but substantially retarded by the treatment on day 7. In the latter case, tumor size regressed and did not increase significantly until day 17, after which growth occurred at a rate approximately parallel to the controls, i.e., the single immunotoxin treatment retarded the onset of growth rather than decreasing the rate of growth. Ghose et al. obtained somewhat similar results in treating melanoma-bearing mice with an MTX-antimelanoma antibody conjugate.[302] In another investigation, a mouse BCL_1 model was adapted to mimic a clinical situation in which patients have a large tumor burden.[459] Affinity-purified ricin A chain was conjugated to the PDT derivative of an anti-idiotypic antibody by disulfide interchange. Purification of the resulting conjugate was by gel filtration and affinity chromatography. The antibody-linked toxin treatment was instituted after reduction of tumor bulk by irradiation and splenectomy. Under these conditions (but not with irradiation alone), it was possible to achieve remission in treated animals, whereas control animals only survived for 7 days. Blood from mice apparently in remission did not produce leukemia when given to normal mice and so did not contain tumor cells. However, blood from long-term survivors was capable of producing tumors under these conditions, indicating that tumor cells persisted in treated animals. It was pointed out that successful immunochemotherapy was dependent on preliminary cytoreduction, given present limits on therapeutic indexes for antibody-linked toxins.

A variety of approaches have been taken to augment the cytotoxicity of A chain toxin conjugates. These include simultaneous exposure to lysosomotropic agents, viruses, or cleaved B chains. The behavior of chloroquine and ammonium chloride in potentiating cytotoxicity of pokeweed antiviral protein and ricin A chain conjugates has already been alluded to. Akiyama et al.[460] have recently reported on the potentia-

tion of cytotoxic activity of *Pseudomonas* exotoxin-antibody conjugates by calcium channel blockers and a lysosomotropic agent. Conjugates were synthesized by the same method and with the same antitransferrin receptor antibody (HB21) as used by FitzGerald et al.[461] (see later) and with EGF. Conditions were chosen so as to give 1:1 conjugates. A colony inhibition assay with KB cells showed that verapamil, D-600, diltiazem, and the lysosomotropic agnet, beta-glycylphenylnaphthylamide, increased cytotoxicity of conjugates in a concentration-dependent manner. For example, D-600 at 20 μg/mℓ gave the largest effect, lowering the I_{50} for inhibition of cell growth from 3 to 0.2 ng/mℓ. These agents alone had no effect on colony formation at the concentrations used. Potentiation effects were usually less on other cell lines. Another assay based on inhibition of protein synthesis measured by tritiated leucine uptake gave results in which the conjugates exhibited less cytotoxicity, but there was greater potentiation by agents. The mechanism of potentiation is not clear. In a previous study by this group,[462] verapamil-treated KB cells delayed lysosomal degradation of ^{125}I-labeled EGF, indicating interference with lysosomal function as an underlying mechanism. Also, hydrolysis of beta-glycylphenylnaphthylamide in lysosomes has been reported to produce a metabolite that damages lysosomal and perhaps other cell membranes.

FitzGerald et al.[461,463] have investigated the ability of human adenovirus type 2 to increase the toxicity of *Pseudomonas* exotoxin conjugated to monoclonal antitransferrin receptor antibodies or EGF. Conjugation was achieved by disulfide interchange.[464] Methyl-4-mercaptobutyrimidate was used to introduce thiol groups into both toxin and carrier protein (an average of two in the toxin and one in the carrier). The modified toxin was treated with 5,5′-dithio-*bis*-nitrobenzoate to produce a thionitrobenzoate derivative, after which conjugation to thiolated carrier protein was carried out by incubation with a threefold molar excess of the thionitrobenzoate derivative. The conjugate was isolated by adsorption on protein A at pH 8 followed by elution at pH 6. ADP ribosylating activity was retained in the conjugate. Exposure of cells in culture followed by measurement of inhibition of tritiated leucine uptake showed that the antibody conjugates had I_{50} values of the order of 0.1 to 0.3 n*M*, whereas the cysteine-substituted toxin was not cytotoxic at 10 n*M*. This control was included because reaction of the toxin with methyl-4-mercaptobutyrimidate abolishes binding to the receptor. This effect on binding indicates that uptake by nontarget cells would not occur if conjugate disulfide linkages were reduced in vivo.[461] Concurrent exposure of KB cell monolayers to conjugate plus adenovirus increased toxicity 100- to 300-fold. Adenovirus alone did not inhibit protein synthesis under the experimental conditions. The cytotoxicity of conjugates depended on the human cell line tested. Earlier studies by Trowbridge and Domingo[465] had shown that ricin A chain conjugated to antitransferrin receptor antibodies was cytotoxic, and FitzGerald et al.[461] found that adenovirus was also able to potentiate the effect on protein synthesis of their ricin A chain-antibody conjugates. It was concluded from immunofluorescence measurements that both conjugate and adenovirus were taken up into the same receptosome, allowing the virus to disrupt the receptosome and so increase release of toxin molecules into the cytosol. A capsid protein of the virus appears to mediate the effect, so the isolated protein might be utilized in combination with antibody conjugates to increase therapeutic effectiveness.

The cytotoxicity of A chain conjugates has been restored by inclusion of free B chains in an in vitro protein synthesis assay system.[374,451,466] The effect was about fivefold and is thought to involve increased efficiency of entry into the cytoplasm rather than increased binding of conjugated A chain.[466,467] Vitetta et al.[374,451] have investigated potentiation of the cytotoxicity of ricin A chain-IgG-antibody conjugates by ricin B-chain-IgG-antibody conjugates, both linked by disulfide interchange. They used affinity-purified rabbit IgG against human Ig since their test system consisted of a target

Burkitt's lymphoma cell line possessing surface IgG. The ternary conjugates were purified by Sephacryl S-200 chromatography and then by affinity chromatography with immobilized human Ig to eliminate residual free A or B chains. Solid phase RIA using ^{125}I-labeled anti-A or -B indicated one to two A or B chains per Ig. In vitro measurements of uptake of tritiated leucine by Daudi cells showed that little toxicity was manifested separately with the A-chain conjugate at 0.3 $\mu g/10^5$ cells or with the B-chain conjugate at levels substantially above this. In contrast, mixtures of the two gave significant toxicity. The effect reached almost an order of magnitude with a mixture at 0.3 (A conjugate) and 2.6 (B conjugate) $\mu g/m\ell$. Synergism with mixtures of free chains and either of the conjugates was found only at high concentrations and was reported to be modest. A and B chains conjugated to irrelevant IgG gave no synergism, with or without galactose. This is indicative of a decrease in B chain-based binding on its conjugation to an Ig. This approach shows that it may be possible to circumvent the nonspecific toxicity associated with whole linked-polypeptide-toxin conjugates without losing the B chain function.

One variation in conjugation is to link the A chain to a univalent F(ab) antibody directed against the target cell surface and the B chain to an anti-Ig antibody.[451] The use of a F(ab) fragment was expected to avoid binding to Fc receptors on nontarget cells and lead to delayed endocytotic uptake, thus allowing the B-chain conjugate to be administered at a later time. The same affinity-purified rabbit antihuman Ig as described above was used as the carrier for the ricin A chain and goat antirabbit Ig antibody was used for the B chain. Incorporations of one to three A or B chains per antibody molecule were obtained with less than 1% contamination by free chains. Cytotoxicity was assayed using sequential 15-min incubations with conjugates followed by 22 hr of growth in conjugate-free medium, after which uptake of tritiated leucine was determined. The B chain linked to antirabbit Ig potentiated cytotoxicity of the cell-surface targeted A chain conjugate in a manner comparable to that observed before with both chains linked to the same antisurface Ig carrier antibody. Synergism was not observed if the B chain was linked to an irrelevant Ig. The conjugate of F(ab) and A chain was equal in cytotoxicity to the corresponding whole Ig conjugate and was also potentiated by B chain linked to antirabbit Ig. The synergistic effect decreased linearly with increase in the time interval between exposure to the A-chain and B-chain conjugates, e.g., half the effect was still present at 5 hr. The authors suggest that in an in vivo context targeted A-chain univalent F(ab) conjugate which did not bind to target cells could be substantially eliminated prior to administration of a B-chain conjugate targeted to the A-chain carrier antibody.

C. Antibody-Bound Radionuclides

Radioisotopes have several characteristics that make them attractive for antibody-mediated targeting against tumor cells. Ionizing radiations are widely used in the treatment of cancer, and factors determining the response of mammalian cells to different types of radiation are fairly well known. Cell damage is caused either by emitted charged particles or quanta of energy and therefore there is no necessity for the isotope to be endocytosed. Binding of the isotope-carrying antibody can deliver radiation to several adjacent cells that may lack the target antigen.[296]

The criteria of suitability of antibody-linked radioisotopes for therapy differ from those for imaging. A high tumor-to-normal tissue ratio of localization is the most important factor in tumor imaging. Furthermore, for conventional external imaging (i.e., methods not based on positron emission), the radioisotopes must be gamma emitters with a short half-life and should be rapidly cleared from the body to reduce radiation hazards. For therapy, high and prolonged concentration of the radioisotope at the tumor site are desirable features and a relatively low tumor-to-normal tissue ratio of

localization may be adequate. Radioisotopes for therapeutic purposes should produce a tumoricidal amount of ionization at the site and the radioactivity should be adequately localized, have low penetration to avoid systemic effects, and remain tumor bound for a period sufficient to deliver a tumoricidal dose. The optimal time to deliver such a dose will depend upon the decay scheme of a given radioisotope. Eckelman et al.[468] have listed the physical properties and pharmacokinetics of some radioisotopes that may be of use in antibody-mediated tumor imaging and therapy.

^{131}I is the most used radionuclide for antibody-targeted therapy of experimental and human cancer.[143,308,469-473] Definite evidence of tumor regression has been obtained only with this radioisotope.[310,469,470] Reasons for its employment in radioimmunotherapy include its established use in the treatment of hyperthyroidism, the availability of several mild methods for iodination of Ig, its physical characteristics, and the range of radiation in tissues. The reaction conditions for iodination of proteins can be manipulated[468] so that it is possible to control the level of incorporation of the isotope.[15] Substantial antibody activity is retained when the level of incorporation of ^{131}I does not exceed two atoms of iodine per molecule of IgG.[300,310,469,474] Higher levels of incorporation led to a decrease in the affinity of the antibody and its faster clearance from serum.[310] The physical characteristics of ^{131}I are such that, in spite of the limited cell surface concentration of TAA, antibodies labeled with less than two atoms of ^{131}I per IgG molecule can deliver a tumoricidal amount of radioactivity.[12,310] The effective beta radiation from ^{131}I has a range in tissue corresponding to several times the diameter of a cell.[296] Although this is likely to eradicate adjacent tumor cells that lack the target antigen and do not bind the labeled antibody, adjacent normal cells can also be damaged. In any case, nonuniform distribution of radioactivity may fail to deliver lethal amounts of radioactivity to all adjacent cells. The features of ^{131}I that limit its usefulness for antitumor targeting include the susceptibility of antibody-bound iodine to dehalogenation in vivo[468] associated with uptake by unblocked thyroid, immuno- and myelosuppressive effects,[310,469] potential carcinogenic effects (not so far observed in adults treated with ^{131}I for hyperthyroidism),[475] and the very rare incidence of allergy to iodine.

Use of radionuclides that emit softer β-rays than ^{131}I or of alpha emitters may achieve more precise and uniform cell kill, but the effective range does not exceed that of a single diameter and adjacent antigen-negative tumor cells are not likely to be affected. Nevertheless, ^{211}At appears to have several desirable features for use in immunoradiotherapy of cancer. The alpha particles emitted during its decay have high average energy (Ea = 6.8 MeV), high linear energy transfer (i.e., induce high specific ionization), and an effective range of several cell diameters. Their effect on cells and tissue is fairly independent of oxygen saturation, and tumoricidal effects in vivo have already been demonstrated.[476] In another approach to immunoradiotherapy of cancer, ^{10}B has been linked to anti-TAA antibodies,[477,478] with the expectation that after exposure to slow neutron radiation thermal neutrons will be captured with the release of 2.79 MeV of energy. However, tissue penetration of thermal neutrons is poor and whether the ^{10}B really adds to the effect of neutron beams alone is still controversial.[479,480]

VI. POTENTIAL APPLICATIONS AND CLINICAL EXPERIENCE

Drug-antibody conjugates and radiolabeled antibodies have been used in preliminary phase I investigations in cancer patients. In general, drug conjugates have shown a more consistent antitumor activity than immunotoxins in tumor-bearing animals. Furthermore, the mode of action and pharmacokinetics of cancer chemotherapeutic agents

and radionuclides are better understood than those of the protein toxins. Finally, as cancer chemotherapeutic agents are widely used clinically, the use of their conjugates entails fewer ethical problems than immunotoxins. This has confined the use of immunotoxins mainly to the elimination of target cells, e.g., leukemia/lymphoma cells or subsets of immunocompetent cells, from bone marrow in vitro prior to bone marrow transplantation.

A. Purging of Neoplastic Cells In Vitro

Autologous bone marrow transplantation is being increasingly used in the management of leukemias and non-Hodgkin's lymphomas.[481,482] Marrow obtained from a patient, even in remission, is likely to contain small numbers of neoplastic cells which may cause relapse of the disease when the marrow is transfused back after supralethal chemoradiotherapy. Selective and complete elimination of contaminating malignant cells from the bone marrow to be retransfused thus constitutes a major problem. Methods that have been used to remove tumor cells from marrow include physical separation,[254] cytotoxic drugs,[483] anti-TAA antibodies together with complement,[116,484-487] MAB linked to intact ricin,[363,378,488] MAB linked to ricin A chain,[439,455,459,489] pokeweed antiviral protein,[373] MAB linked to chemotherapeutic agents,[490] anti-TAA MAB and chemotherapeutic agents unlinked,[491] or the combination of a chemotherapeutic agent and immunotoxin.[492] Only very preliminary clinical evaluation of some of these methods for purifying bone marrow in vitro has been reported, e.g., chemotherapeutic agents,[493,496] antibodies along with complement,[495-497] or immunotoxins.[498] Antibodies and complement tend to be toxic towards progenitor and other non-neoplastic cells in the marrow, cause clumping of cells, and lead to antigenic modulation.[499] Furthermore, many MAB do not fix complement or require very high antibody concentrations for cell killing.[118] Exposure to multiple antibodies has been demonstrated to be more effective in eliminating neoplastic cells than the use of a single anti-TAA antibody.[487,497] In contrast to the short time needed for the killing of cells by complement and antibody, immunotoxins need hours or days to kill target cells.[500]

Of interest are a number of model studies with immunotoxins aimed at eliminating specific cell types from bone marrow ex vivo. The studies of Uckun et al. with conjugates of pokeweed antiviral protein were discussed previously.[373] In an experiment to simulate eradication of malignant cells from human bone marrow, a mixture of bone marrow and 15% BCL_1 leukemic cells was treated in vitro with ricin A chain conjugated by disulfide interchange to an anti-BCL_1 idiotypic Ig and then adoptively transferred to lethally irradiated mice. There was selective but not total eradication of tumor cells with a loss of no more than 10% of normal bone marrow cells.[29] Another example comes from the studies of Muirhead et al.,[439] who conjugated ricin A chain to an affinity-purified polyclonal Ig against human lambda and kappa chains by disulfide interchange. This conjugate was able to eliminate 99% of surface IgM/kappa-bearing Daudi cells mixed with marrow cells in vitro without demonstrable toxicity to hemopoietic cells.

Several studies have also been carried out to eliminate leukemic T-cells from human bone marrow. Casellas et al.[501] produced a ricin A-chain immunotoxin using the method of Jansen et al.[502] with MAB T101, an IgG2a against the T1 65-kdalton glycoprotein differentiation antigen expressed by T-cells and CLL cells but not by normal myeloid and erythroid progenitor cells. Assay of the conjugate in a cell-free protein synthesis system showed that 1.5 to 2 mol of active A chain was present per mole of IgG2a. Fluorescence assay showed that all antibody activity was retained. In a clonogenic assay, CEM cells treated with the specific conjugate at 10 n*M* in the absence of ammonium chloride exhibited 14% survival at 24 hr. At this conjugate concentration,

substantially all target antigen sites were occupied when cell numbers did not exceed 2×10^7 mℓ. Almost all CEM cells (99.99%) treated with the specific conjugate plus 10 m*M* ammonium chloride were killed at 8 hr. (The more rapid killing produced by the ammonium chloride should improve clinical efficacy.) Cells which survived the treatment with conjugate plus ammonium chloride lacked antigen. One million cells from an antigen-positive subclone were all killed by the specific toxin under identical experimental conditions, indicative of a 6-log or better efficacy factor. This treatment allowed survival of hematopoietic progenitor cells. Cytotoxicity was blocked by unconjugated antibody. A conjugate prepared with anti-CEA antibody was without cytotoxic effect. The T1-negative cell lines, Daudi and Raji, were not inhibited by the T101 conjugate up to 10 n*M*, although their sensitivity to ricin or ricin A chain is similar to that of CEM cells. An advantage in clinical application pointed out for A chain conjugates as opposed to whole ricin conjugates was the fact that the treated marrow would not have to be carefully washed to eliminate residual whole toxin before re-infusion into a patient.

An in vitro T-cell leukemic model was used to investigate conjugates of whole ricin toxin prepared by disulfide interchange.[378] The antibodies were T101, which gave the most effective conjugate, and 3A1, an IgG1 which recognizes a 4-kdalton antigen on T and some early myeloid cells. Residual free ricin was removed from the product by protein A-Sepharose chromatography and free antibody by Sepharose 4B chromatography. Retention of toxin activity in the conjugates was confirmed by a cell-free protein synthesis assay. Either specific conjugate strongly inhibited antigen-bearing CEM and 8402 ALL cell lines by approximately 2 logs at a concentration of 0.1 n*M* but not a B-cell leukemia (8392) or promyelocytic leukemia (HL60) cell line, although colony formation by bone marrow progenitor cells was inhibited approximately 30% at this concentration. The cytotoxic effect of the T101 conjugate toward CEM cells was maintained in mixtures of CEM and bone marrow progenitor cells. Release of free ricin from treated cells did not occur in sufficient amounts to present a clinical hazard. The results of this study, as well as that of Stong et al.,[488] showed that the effectiveness of whole ricin-antibody conjugates was not solely determined by the amount bound to the cell surface, probably because of problems with internalization.[378]

Immunotoxins constructed with ricin A chain were more potent when two or more conjugates directed against independent epitopes were combined.[503,504] In a study using immunotoxins constructed with intact ricin and four MAB against independent epitopes on leukemic T-cells from acute T-cell leukemia patients (i.e., MAB T101, G 3.7, 35.1, and TA-1), the immediate toxicity (protein synthesis inhibition) was compared with the inhibition of clonogenicity by these conjugates. Inhibition of protein synthesis in the presence of lactose in vitro correlated well with the extent of antigenic expression on the surface of the leukemia cells. The conjugate constructed with T101 gave the fastest rate of protein inhibition. A cocktail of four cell conjugates exhibited kinetics that were as fast or faster than the kinetics of the T101 conjugate. On colony inhibition assay in the presence of lactose, all four immunotoxins were specifically cytotoxic and a cocktail of the four was more potent than the T101 conjugate alone.[500] In a phase-I trial, a ricin A chain T101 conjugate was used to purge autologous bone marrow of malignant T-cells. In every patient, hematopoetic recovery occurred within normal time periods, but two patients developed severe infection. There was a suggestion of increased fragility of the incubated progenitor cells to freezing.[498,505]

Another approach to potentiating the action of immunotoxins in vitro is addition of ammonium chloride and/or mafusamid or chloroquine during incubation.[373,492,503,505] Douay et al.[505] demonstrated that an MAB T101-ricin A chain conjugate in the presence of ammonium chloride could eliminate 99.9% of target cells without interfering with the proliferative capacity of hematopoietic stem cells. Ricin (10^{-7} *M*) linked to a

MAB and incubated in vitro in the presence of ammonium chloride could eliminate at least 4 logs of clonogenic tumor cells from a 20-fold excess of bone marrow.[503] This is comparable to the tumor cell kill achieved with 4-hydroxyperoxycyclophosphamide (see below) or with multiple MAB in the presence of complement. Uckun and coworkers[453] have reported that mafusamid along with a ricin-anti P67 MAB conjugate specific for both normal and leukemic T-cells[453] or with a ricin-PAN-B MAB conjugate[492] could eliminate on the order of 7 logs of target cells with minimal toxicity to normal bone marrow progenitor cells. These studies are important because to be useful for the removal of neoplastic cells from bone marrow the carrier MAB should not react with hematopoietic stem cells.

The efficiency of anti-TAA antibodies and their conjugates in eliminating tumor cells from bone marrow will depend upon the variability of expression of the target TAA, the heterogeneity of TAA, and the specificity of the antibody, i.e., the extent of its reactivity with hematopoetic progenitor cells. Several MAB have been compared as regards their efficiency in removing malignant B cells from bone marrow.[503] It is interesting that the efficiency of several anti-B-cell MAB for complement-mediated lysis did not correlate with their effectiveness as a component of immunotoxins.

It has been claimed that HLA-DQ determinants are expressed on mature malignant lymphoma cells but not on normal bone marrow progenitor cells.[506] A panel of polyclonal and monoclonal anti-HLA-DQ antibodies in the presence of complement has been reported to be able to eliminate 98% of clonogenic tumor cells in vitro. Immunotoxins based on these antibodies have not yet been evaluated.

B. Purging of Immunocompetent Cells from Bone Marrow to Prevent Graft-Vs.-Host Disease (GVHD)

Another use of antibody-linked agents in the management of human leukemias is the prevention of GVHD, which develops in approximately 50% of patients given bone marrow grafts from HLA-matched siblings.[482] Attempts are being made to control GVHD by in vitro depletion of T-cells from grafts prior to transplantation by immunophysical methods,[507] by administration of a cocktail of anti-T-cell MAB,[482,508] or immunotoxins.[509-511] In a pilot clinical study, 14 patients were given bone marrow treated in vitro with an immunotoxin constructed with intact ricin bound to three different, well-characterized anti-T-cell MAB. In vitro analysis of treated marrow samples revealed significant T-cell depletion but sparing of hematopoetic cells. No toxicity could be seen in the patients transfused with the treated bone marrow. According to these investigators,[510] there was prompt acceptance of the grafted bone marrow in their patients. In another study based on eight patients given autologous bone marrow, there was considerable reduction of the risk of developing GVHD when mature T-cells were removed from the bone marrow graft with anti-T12 MAB and complement.[497] However, there is evidence from other studies that the acceptance of bone marrow grafts is inhibited after T-cell depletion.[512] In regard to patients with developed GVHD, 15 patients with steroid-resistant severe GVHD were given various doses of one or more anti-T-cell MAB (a total of four MAB against independent epitopes was used). Most of the patients treated with MAB needed platelet infusions. However, the amount of platelets needed was not different from that needed by patients subjected to other therapeutic regimens. Six of ten patients given intermediate doses of the MAB had evidence of at least partial improvement. None of the patients developed detectable evidence of immunization with mouse Ig. Apart from fever and chills, there was no evidence of toxicity.[513]

C. Preliminary Experience with Drug Antibody Conjugates in Patients

1. Melanoma

In a study by Ghose et al.,[276] 13 consecutive patients with inoperable recurrent malignant melanoma were treated with chlorambucil bound to goat or rabbit antihuman melanoma Ig. The next consecutive 11 patients fulfilling the criteria for admission into this study were treated with chemotherapy only, i.e., dimethyltriazenoimidazole carboxamide (DTIC). Follow-up was for a minimum of 29 months or until death. Two patients showing an objective response to immunochemotherapy had disease confined to lymph nodes and cutaneous sites; five others showed stabilization of cutaneous, nodal, and visceral metastases; and six patients showed progression of their disease. The median survival of the responders and stabilizers was 20 months, but only 3.5 months for patients with disease progression. None of the 11 patients treated with DTIC had objective tumor regression and all died within 11 months of the start of treatment, with a median survival of 3 months. Immunochemotherapy significantly prolonged survival compared to that in the DTIC-treated group ($p < 0.05$). No hematological or renal toxicity was detected after immunochemotherapy.

Recently, 3 of 12 melanoma patients given an IgG_3 mouse MAB against the GD_3 antigen showed "major" tumor regression. Patients given more than 80 mg/m^2 of the antibody had an inflammatory reaction at tumor sites. Examination of biopsied tumor tissue revealed complement deposition, infiltration of mast cells and lymphocytes, and mast cell degranulation.[497] A phase-I trial of an immunotoxin constructed with ricin A chain has also been completed. It has been reported that there was no major problem with toxicity, contraindicating the clinical use of the immunotoxins. Three main toxicities observed in this trial were lethargy and malaise, evening fever, and reversible hypoalbuminemia without proteinuria.[514]

2. Neuroblastoma

Melino and co-workers have treated a number of neuroblastoma patients with antibody-conjugated drugs.[515-519] The carriers were allogenic polyclonal antineuroblastoma antibodies prepared by immunizing haploidentical volunteers with irradiated neuroblastoma cells and fractionation of plasma with cold ethanol.[517] One antibody reacted with seven out of eight neuroblastoma cell lines tested.[516] Preparations could be obtained that did not contain anti-HLA antibodies or cross-react with normal human brain.[516] Conjugation of either daunorubicin or chlorambucil was carried out using a water-soluble carbodiimide. A total of 3 to 4 mol of drug was incorporated per mole of Ig. SDS-PAGE showed polymers, but both daunorubicin and chlorambucil conjugates were reported to retain essentially full antineuroblastoma activity as determined by membrane immunofluorescence.[517] In a cytotoxicity assay using human neuroblastoma cells exposed in vitro to a conjugated daunorubicin concentration of 20 μg/mℓ, the conjugate produced a 60% kill compared with 35% for a mixture of free drug and antibody.[515] Two patients treated with daunorubicin conjugates showed good initial responses, then relapsed and died at 3 and 8 months. Four remained alive and disease-free after 6 to 19 months of treatment. No drug side effects were observed.[515] They later reported that 9 of 12 patients treated with conjugates of both daunorubicin and chlorambucil (30 mg IgG/kg, twice a week) showed marked "antitumor responses" with no detectable anti-idiotypic or antiallotypic antibodies or other blocking factors.[516] No toxic side effects were noted. A recent report from this group[517] dealt with seven patients with advanced neuroblastoma treated following a protocol consisting of one conjugated chlorambucil (0.5 mg/kg) and two conjugated daunorubicin (1 mg/kg) injections per week for 1 year. Lower catecholamine levels were noted in 7 out of 7 patients. Partial regression of tumors occurred in patients with stage IV disease, while those with less than stage IV cancer had no evidence of disease after 3 years.

In a single patient with metastatic disease who had prior chemotherapy and surgery, treatment with conjugated chlorambucil alone and then with conjugated chlorambucil and daunorubicin led to initial regression of lesions followed by resumption of growth. The lack of therapeutic response was attributed to drug resistance since the antibody bound to tumor from the recurrent primary removed at operation.[519]

VII. MODE OF ACTION

Although knowledge of the mode of action of anti-TAA antibody conjugated agents should contribute to the design of more effective conjugates, this area of investigation is only now accelerating; the focus hitherto has been on documenting cytotoxicity in vitro and in vivo. In investigating the mode of action, it is necessary to consider: (1) the antitumor effect of an antibody alone; (2) additive or synergistic effects of antibody and cytotoxic agent unlinked; and (3) alterations in the activity of an agent and in its pharmacokinetics as a result of linkage to antibody.

A. Effect of Antibodies Alone on Tumor Growth In Vivo

The experience of Ghose et al.[520,521] and that of others[491,515-524] shows that only a very small tumor load of 10^5 to 10^6 cells can be eradicated by passive serotherapy with polyclonal anti-TAA antibody. Limitations of conventional antitumor sera include: (1) difficulty in raising amounts adequate for therapy; (2) relatively low titer due to repeated absorptions with normal tissues for obtaining tumor specificity; and (3) persistence of nonspecific Ig. However, increasing numbers of reports are appearing on MAB serotherapy of experimental and human tumors, especially leukemia and lymphoma, as summarized by Ritz and Schlossman.[118] Also, a number of phase I feasibility studies with MAB in human cancer have been listed by Dillman and Royston.[525] Ideally, antitumor MAB should react with the target tumor only, but so far, MAB that react with tumor as well as some normal cells (and are not significantly toxic to the recipient) have been the ones most used in human serotherapy. Treatment in humans involved different types of leukemias and lymphomas with MAB of different specificities[118] and melanomas with MAB against P97, GD_3, and a proteoglycan antigen.[526,527] With respect to B-cell CLL, Dillman et al.[314] treated two CLL patients with MAB T101 directed against a 65-kdalton protein expressed by normal and malignant T- and B-cells. Administration of the antibody resulted in rapid clearance of circulating leukemia cells, but they soon returned to pretreatment levels in both patients. Both had adverse reactions to the MAB infusion. On the other hand, Miller et al.,[312] using a monoclonal anti-idiotypic antibody, induced a dramatic response without any acute or chronic toxicity in a patient with B-cell lymphoma. "Major tumor regression" occurred in 3 of 12 melanoma patients treated with MAB R_{24} against GD_3[527] but in none of the 5 patients treated with the other two MAB.[526] However, generally the results of clinical trials of anti-TAA antibodies alone in cancer patients have been disappointing.[118] It should be realized that antibodies themselves are not inherently cytotoxic and their binding to a target cell membrane does not affect growth except in rare instances.[12] Possible mechanisms by which they could produce tumor inhibition in vivo include complement (C)-mediated cytotoxicity, clearance by reticuloendothelial cells, and antibody-dependent cell-mediated cytotoxicity.[525] Investigations into the mechanism of tumor inhibition by MAB have revealed that those of the IgG class inhibit tumor cells by C-dependent cytotoxicity and antibody-dependent cell-mediated cytotoxicity, whereas MAB of the IgM class inhibit tumors only by C-dependent cytotoxicity. IgM anti-TAA MAB have failed to show any effect on tumor growth in vivo.[118,301] This suggests that C-dependent cytotoxic mechanisms do not contribute in a major way to tumor inhibition. This is consistent with our observations using conventional xenogenic sera.[520] In leukemia pa-

tients, administration of MAB is followed by a rapid fall in circulating leukemic cells due to their sequestration in the reticuloendothelial system. However, this reduction is only transient.

In summary, the results of serotherapy with anti-TAA MAB in experimental and human cancer show transient tumor suppression, a modest increase in survival, and eradication of an individual tumor burden of approximately 10^5 cells. The magnitude of the clinical problem can be appreciated by the fact that the tumor burden is very high in patients refractory to standard therapy (e.g., 10^{12} cells). In the patient described by Miller, the infusion of 1 mg of MAB removed approximately 2×10^{11} tumor cells, yet the influence on progression of the disease was negligible, i.e., the total tumor cell population recovered in 24 hr.[312] It is, therefore, essential to increase the cytodestructive effect of anti-TAA antibodies, e.g., by their linkage to cytotoxic agents.

B. Additive or Synergistic Effects of Cytotoxic Agents and Antibodies Unlinked

Synergistic tumor inhibitory effects of anti-TAA antibodies plus cancer chemotherapeutic agents have been demonstrated both in vitro[528,529] and in vivo.[530] Ghose et al. confirmed synergism with chlorambucil,[395,398] MTX,[345] and Trenimon[292] in tumor-inoculated mice. However, the full extent of tumor inhibition by cytotoxic agent-anti-TAA antibody conjugates cannot be explained entirely by synergism,[395,398] except that with the alkylating agent Trenimon there was no difference in the extent of tumor inhibition by Trenimon linked to anti-TAA antibody and equivalent amounts of the drug linked to a nonspecific IgG together with the anti-TAA antibody unlinked.[292]

Exposure of tumor cells to certain cancer chemotherapeutic agents or metabolic inhibitors has been shown to inhibit the synthesis of complex cell surface lipids and render them more susceptible to antibody and complement.[531] If the toxic moiety in conjugates is surface active, then the agent and antibody may act additively or synergistically. Damage to the cell membrane by the conjugate or its components may also facilitate entry into cells and allow access to intracellular targets. Potentiation of the antitumor effect of ionizing radiations by anti-TAA antibodies has been observed both in vitro[532] and in vivo.[469] Ionizing radiations may also interfere with the permeability of plasma membranes.[533]

C. Alterations in the Action of an Agent and in Its Pharmacokinetics as a Result of Linkage to Antibody

The therapeutic index is the critical factor in the application of antibody-conjugated agents. There may well be a decrease in drug activity in a conjugate compared to the free agent, but the systemic toxicity of that conjugate may be lower than that of the free drug, allowing a compensating higher dose of conjugated drug. Gallego et al.[334] have emphasized that tumor-selective cytotoxicity in vivo is a more important parameter than cytotoxicity in vitro.

One approach to an insight into the mode of action of antibody-linked cytotoxic agents is to study and compare the effect of intact conjugates and their components (especially the cytotoxic entity): (1) at the molecular level by investigating interaction with putative target molecules of the agent in cell-free system, (2) at the cellular level by investigating effects of cells in culture, and (3) at the level of intact animals by investigating effects on tumor-bearing animals or cancer patients. In evaluating the mechanism of action on tumor cells in culture, factors that need consideration include: (1) access of agents, free and conjugated, to target molecules, especially if these are intracellular; (2) the catabolism of conjugates and their components during transit to target molecules; and (3) the removal (e.g., efflux or otherwise) of pharmacologically active moieties from the target compartment. In intact animals, additional considerations include: (1) stability, especially the retention of agent and antibody activities in

conjugates while in the circulation; (2) factors that govern homing and catabolism during transit from the vascular to the extravascular compartment in solid tumors; and (3) alterations in the pharmacokinetics and systemic toxicity of agents as a result of conjugation. Although somewhat sketchy, information at these three levels is gradually emerging. In particular, conjugates containing MTX, adriamycin/daunorubicin, or chlorambucil and immunotoxins constructed with the A chain of ricin have been investigated.

1. Action at the Molecular Level

Linkage of cytotoxic agents to Ig or other macromolecular carriers may either augment or inhibit the effect of the agent on its target molecules. For example, linkage of chlorambucil to Ig inhibits its catabolism or transformation to inactive forms[297] and thus preserves alkylating activity, which is accepted as the basis of its cytotoxic action. On the other hand, there may be loss of activity of the agent during or after conjugation due to various causes that include inappropriate reaction conditions leading to chemical damage of the agent, e.g., loss of activity of MTX during conjugation following the "mixed anhydride" method[345] and steric hindrance. Ghose et al. observed that adriamycin conjugated to IgG or high molecular weight dextran does not bind to DNA in vitro.[13] Intercalation into DNA is thought to be essential for the expression of the cytotoxic effect of these agents.[12] The elegant studies of Trouet et al.,[339] Monsigny et al.,[338] and Shen and Ryser[333] have demonstrated that intralysosomal release of daunorubicin can restore the cytotoxic action of inert conjugates. Ghose et al. also demonstrated that MTX conjugated to IgG or its $F(ab')_2$ moiety is only half as potent in inhibiting its target enzyme DHFR as the free drug.[299,345] In regard to immunotoxins, it has been demonstrated that intact diphtheria toxin or ricin inhibits protein synthesis in cell-free systems at least 1000-fold less effectively than the free A chains. Thus lysosomal cleavage of the A chain appears to be essential even though the mode of transport of the cleaved A chain to the cytosol is not well understood.[534,535] Surprisingly, conjugates in which the A chain of ricin was linked to the F(ab) moiety of IgG were as effective inhibitors of protein synthesis in cell-free systems as the free A chain, irrespective of whether the linkage between the A chain and F(ab) was cleavable.[536] The difference in the behavior of B chain-linked and F(ab)-linked A chains of ricin in cell-free systems has been attributed to the inflexibility of the covalent and noncovalent bonds between A and B chains. It should be stated that antibody or other macromolecule-linked agents may exert their cytotoxic effect via mechanisms that are not usual or predominant with the free agent. Thus intracellular DNA is regarded as the principal target of alkylating agents like Trenimon and chlorambucil or intercalating agents like adriamycin and daunorubicin. However, all these agents are also surface active and conjugates containing them could damage cell membranes and be cytotoxic even in the absence of endocytosis.[292,537]

2. Action on Target Cells In Vitro

A large number of antibody conjugates have been produced and the specificity of their binding to target cells in vitro has been demonstrated.[14] However, on comparing the biological effectiveness of antibody-linked agents with that of the agent, free or linked to nonspecific Ig, two patterns of inhibition of target cells in vitro have been observed. In some experiments, the order of tumor inhibition has been free drug > drug linked to anti-TAA antibody > drug linked to nonspecific Ig. This pattern has been reported with Trenimon,[292,538] MTX,[345] purothionin,[409] adriamycin,[13,264] and vindesine.[431] The other pattern of inhibition is antibody-linked agents > free drug > or = drug linked to nonspecific Ig, and has been observed with other drugs such as

chlorambucil[394] and with drugs in the first group but assayed by different methods on different cell lines, e.g., daunorubicin[415,416,539] and vindesine.[430] This observed difference in the relative effectiveness of conjugates may be due to differences in the carrier antibody and its target antigen, the nature of the agent, especially its site of action and its transport characteristics, the method of linkage, the cell line used, and the method used for assaying tumor inhibition. For example, Embleton et al.[431] have observed that when the target cells were exposed to vindesine and its conjugates for 24 hr free drug was 2000 times more cytotoxic than the antibody conjugate; with shorter exposure the free drug was far less effective. For agents that need to be internalized to exert cytotoxic effects, it is essential that the carrier antibody is capable of being endocytosed after binding to its receptor on the cell surface. Endocytosis can follow capping of the cell surface-bound antibody conjugate,[297] although this may not be always so.[292]

For the construction of tumor-specific immunotoxins, it is essential either to cleave off or inhibit indiscriminate binding via the B chain to various nonmalignant cells.[340] Since the target molecules of most of the protein toxins are intracellular,[14,340] binding of immunotoxins to the surface of target cells with subsequent endocytosis is a prerequisite for their cytotoxic action. It is, therefore, not surprising that most of the immunotoxins without B chains show exquisite specificity of binding to tumor cells in vitro and the cytotoxic potency that is higher by several magnitudes than that of the free A chain or A chain linked to a nonspecific carrier. The latter two are endocytosed very poorly by most tumor lines in vitro.[540] It should be stressed that irrespective of whether the activity of antibody conjugates exceeds that of the free drug, all agents have been rendered tumor specific after linkage to anti-TAA antibodies, i.e., antibody-linked agents have been shown to be more inhibitory towards target cells than towards nontarget cells and more potent toward target cells than equivalent amounts of the agent linked to nonspecific Ig.[14,340]

The sequence of events following the binding of antibody conjugates to the surface of target cells in vitro and the path of uptake of antibody-linked chemotherapeutic agents have been elucidated by a number of elegant studies. Using appropriately labeled daunorubicin and its conjugates, Arnon has observed that the order of rate of uptake and amount of drug taken up by cells and nuclei was free drug > drug bound directly to the antibody > drug bound to antibody via a dextran bridge > drug bound to nonspecific Ig.[539] As already stated, this drug needs to be cleaved off the carrier in lysosomes to be cytotoxic.[333,339] It is possible that the slow release of the active drug from the inert intracellular conjugate constitutes an "intracellular depot" effect adding to the potency of the conjugated agent.

Studies by Uadia et al.[295,307,408] have contributed to the understanding of the mechanism of action of MTX-antibody conjugates. When mouse EL4 lymphoma cells were incubated in vitro, the rate at which the tumor cells took up MTX conjugated with an anti-TAA antibody was much slower than the rate of uptake of free MTX. However, as early as at 6 hr of incubation, the net uptake of conjugated MTX exceeded that of the free drug (or drug linked to nonspecific Ig). Furthermore, when human melanoma M21 cells were incubated in vitro with MTX, free or conjugated to several different anti-TAA antibodies, the amount of uptake of conjugated MTX corresponded to the amount bound at equilibrium at 0°C and the titer of the antibody. At 6 hr, more MTX was endocytosed when linked to antibodies than when linked to a nonspecific Ig or free. Furthermore, the efflux of conjugated MTX was much slower than that of free MTX, resulting in the maintenance of prolonged high intracellular levels of MTX. It had previously been demonstrated that more chlorambucil was taken up by EL4 cells in vitro when the drug was conjugated to an antibody than when conjugated to a nonspecific IgG.[541] These results show that more cancer chemotherapeutic agents are endocytosed by tumor cells when they are linked to an antibody than to a nonspecific

protein. At least with MTX-antibody conjugates, the amount of drug taken up as the conjugate exceeds the uptake of the free drug. Some tumors such as the EL4 lymphoma cells show high endocytotic activity and can take up and retain higher amounts of MTX linked to nonspecific Ig than the free drug.[295]

To elucidate the uptake of immunotoxins, FitzGerald et al.[461] designed a study using KB cells and *Pseudomonas* exotoxin conjugated to an antitransferrin antibody. They demonstrated binding of the intact conjugate first to the cell surface with "clustering" and then prominent localization in the coated pits. This was followed by the rapid transit of all the components of the conjugate to receptosomes. The uptake of the conjugate was inhibited by free antibody but not by the free toxin, confirming antibody-mediated endocytosis. Using a ricin A chain immunotoxin adsorbed on colloidal gold particles, Carriere et al.[542] observed internalization of conjugates via either coated pits or noncoated microinvaginations. Conjugates reached receptosomes of lymphoid cells within 15 min of incubation at 37°C and 50% of the intracellular immunotoxin reached lysosomes within 30 min. Apart from the ultrastructural studies on the uptake of immunotoxins, insight into their mechanism of action has been obtained from studies of kinetics of cytotoxic action and on the observed potentiation of their action by lysosomotropic amines and carboxylic ionophores such as monensin.[543] In a study on the kinetics of cytotoxicity produced by intact ricin and immunotoxins consisting of the A chain linked to antibodies against several cell surface antigens, it has been observed that there is usually a lag between exposure of cells to immunotoxins and the beginning of inhibition of protein synthesis. This suggests that immunotoxins must be processed after endocytosis to gain access to target enzymes in the cytosol.[543] It has already been stated that in cell-free systems, the activity of the A chain of protein toxins is lowered when it is linked to the B chain. Lysosomal processing and liberation of A chains from the toxin molecule is thus essential for the full expression of the cytotoxic potential of the A chain. This is consistent with the observation of Masuho et al.[536] that ricin A chain linked to F(ab) moieties by mercaptoethanol-susceptible bonds was more cytotoxic in culture than when linked via mercaptoethanol-resistant bonds. The released A chain may have greater access to the cytosol compartment. As already stated, the A chain linked to an F(ab) moiety was as effective in inhibiting protein synthesis in cell-free systems as the free A chain. This indicates that release of the A chain from the immunotoxin is not important at the ribosomal level, but the A chain must be directed to the ribosome after binding of the conjugates to the cell surface. In most in vitro studies, the rate of inhibition of protein synthesis in exposed cells was much slower with immunotoxins than with intact ricin. This would indicate that the B chain favors special receptors allowing rapid entry or that the B chain facilitates the transport of the A chain into the cytosol compartment. The demonstration by McIntosh et al.[467] that the addition of free B chains potentiates the action of cell-bound antibody-ricin A chain conjugates (which by themselves were not effective), even when the B chain itself does not bind to the cell surface, suggests that a weak noncovalent linkage of the B chain to the cell-bound conjugate facilitates the internalization of the complex. The studies on the kinetics of action of immunotoxins further revealed that the rate of inhibition of the intracellular target enzymes depended upon the number of toxin molecules bound to the surface. The nature of the carrier moiety of the A chain that binds to the cell surface and of the receptors (i.e., antigen) on the cell surface is also important in determining the potency of conjugates. For example, immunotoxins constructed with IgG antibodies were more effective than those constructed with IgM antibodies of identical specificity.[543] Furthermore, A chains linked to $F(ab')_2$ moieties are more cytotoxic than those linked to F(ab).[536] This may be due to greater ease of capping or other modes of internalization of the bound A chain. In none of these studies was there evidence that any significant amount of toxin[542] or drug

moiety[295,539] was cleaved off the cell-bound conjugate and then transported inside the cell using antibody-independent transport mechanisms.

The potentiation of the action of immunotoxins by lysosomotropic amines and monensin has also been of help in elucidating the mechanism of action. Their effectiveness may be based on either alteration of traffic between intracellular compartments allowing the A chain greater access to the cytosol or inhibition of degradation of the A chain in the lysosomal compartment.[542,543] The role of lysosomal processing in potentiating the action of conjugates appears to vary from one conjugate to another. It is possible that fully potent daunorubicin is released from relatively inert conjugates by lysosomal digestion. However, in the case of MTX, Ghose et al. failed to detect any catabolic fragment that is more active than the parent MTX-IgG conjugate.[295] With immunotoxins, even after the release of A chain, it appears that there is the need for appropriate control of the intracellular traffic so that the free A chain gains access to the cytosol compartment.[543] Prevention of excessive and inappropriate catabolism in the lysosomal compartment is obviously also important.

3. Action In Vivo

Tumor-selective localization of the carrier anti-TAA antibody is the basis of antibody-targeted drug therapy. Specific accretion of anti-TAA antibodies, especially MAB and their reactive fragments, has now been demonstrated in many tumor-bearing animals and cancer patients (see above). However, there are only a few studies in which antibody-mediated tumor localization of the bound cytotoxic agents has been adequately investigated in vivo. The study of Uadia et al. with ascites EL4 lymphoma-bearing mice confirmed higher tumor uptake of an antibody conjugate in vivo.[307] For example, at 2 hr after administration, the uptake of MTX given as the antibody conjugate was 2.5 times the uptake of MTX given as a nonspecific Ig conjugate and 6 times the uptake of free MTX. MTX-conjugated to Ig, irrespective of specificity, was retained in all tissues including EL4 cells much longer than free MTX, and MTX conjugated to anti-EL4 IgG was retained longest in the tumor cells. The levels of intracellular MTX after the administration of free or conjugated MTX exceeded the intracellular level of DHFR. Thus, the greater uptake of antibody-conjugated MTX and its prolonged retention by tumor cells in vivo could explain the therapeutic effectiveness of MTX-antibody conjugates in EL4 lymphoma-bearing mice.[345] More interestingly, the amounts of MTX taken up by EL4 cells in vitro correlated well with its uptake in vivo and with the therapeutic effectiveness of the test agents, i.e., antibody conjugate > nonspecific Ig conjugate > free drug. The therapeutic effectiveness of the test agents in melanoma xenograft-bearing nude mice also followed the order of in vitro uptake of MTX, free or conjugated.[302] In a previous study, Ghose et al. demonstrated tumor-selective localization of these antibodies in human melanoma xenograft-bearing nude mice.[139]

These and other studies[540] on the distribution of cytotoxic agent-antibody conjugates in vivo have demonstrated that linkage of agents to macromolecular carriers alters their pharmacokinetics. As already stated, in EL4 lymphoma-bearing mice, Ig-conjugated MTX was cleared slowly and persisted in all tissues including blood, whereas free MTX declined rapidly after reaching peak levels at 1 hr. If the conjugated agent is active, such prolonged elevated levels of the conjugate in normal tissues (and tumor) may account for the increase in nonspecific cytocidal effect in vivo (i.e., increased systemic toxicity) observed with MTX-containing conjugates. Furthermore, prolonged slow release of the agent from the conjugate can also add to its cytotoxicity, especially if the released agent is more potent than the parent conjugate (i.e., the so-called depot effect). Tumor cells that have high rates of endocytosis of serum proteins[544] are likely to be selectively damaged by such nonspecific conjugates. However, linkage of other

agents to Ig has led to a reduction in their systemic toxicity, e.g., Trenimon,[292] adriamycin,[13] and daunorubicin.[539] The reasons for the reduction in the systemic toxicity of conjugated adriamycin or daunorubicin would include the inability of the parent conjugate to bind to DNA and the necessity for lysosomal processing for potentiation. Also, altered physicochemical properties (e.g., hydrophobicity) of the conjugate may prevent access of the conjugated drug to target molecules in myocardial cells, the susceptibility of which limits the dose of these drugs. It is also possible that the rate and extent of activation of conjugated adriamycin/daunorubicin is lower in myocardial cells. The reduction in the systemic toxicity of conjugated Trenimon is not due to the reduction in its alkylating activity because free Trenimon is more potent in vitro than equivalent amounts of Ig-linked active Trenimon, i.e., Trenimon retaining equivalent amounts of alkylating activity which is the basis of its cytotoxic action.[292] Ghose et al.[292] and Linford and Froese[538] have observed that Ig-bound Trenimon is not endocytosed in vitro. This may explain the relative lack of a cytotoxic effect of the conjugate in vitro and its low systemic toxicity. In any case, reduction in the systemic toxicity of these agents in conjugate form allows their administration in higher doses[264,292,539] that can cause tumor inhibition by the stipulated or alternate mechanisms of action (e.g., membrane damage).

In studying the mechanisms of action of antibody conjugates in vivo, one should also consider other factors that determine the ability of the conjugates to home to tumor cells so that they can exert their effect on or inside the target cells. These include stability of the conjugates in circulating blood, their rate of clearance, and retention of antibody activity in the carrier moiety. The factors that determine the clearance of carrier antibodies have already been discussed (see above). The results of a recent study[540] on the pharmacokinetics of an immunotoxin in rabbits are in agreement with those of Uadia et al.[295] in EL4 lymphoma-bearing mice. These studies show that antibody conjugates are fairly stable in circulation and retain both drug and antibody activities and their initial half-life in circulation is fairly long. The factors that determine tumor-selective localization of antibody conjugates will include: (1) the size and charge of the conjugate; (2) its capacity to cross capillary walls; (3) its rate of diffusion and susceptibility to degradation in the tissue space; (4) the presence or otherwise of the Fc moiety of Ig and the B chain of toxins; (5) the specificity of the carrier antibody; (6) the presence in the circulation of TAA, competing antibodies, and immune complexes; and (7) tumor vascularity. These have been discussed previously. Vascular changes in tumors, e.g., large numbers of newly formed vessels, associated increased vascular permeability, and impaired drainage, may also allow intratumoral localization of conjugates in amounts higher than in normal tissues. This may explain the antitumor effect of agents linked to nonspecific Ig and of daunorubicin-antifibrinogen antibody observed by Lee et al.[413,414]

When one considers the alterations in the pharmacokinetic properties of agents when linked to Ig and the factors that determine the transport of active conjugates from the vascular compartment to the milieu of cells in solid tumors, it is not surprising that there are wide discrepancies between the potency of conjugates on cells in vitro and in vivo. For example, with Trenimon, MTX, and adriamycin, the conjugates were consistently less potent than equivalent amounts of free drugs in vitro. In contrast, in tumor-bearing mice, conjugates were by far the most effective. Even nonspecific Ig conjugates, which were usually least effective in vitro, were more potent than the free drug in vivo. The factors that contribute to the effectiveness of nonspecific Ig-bound agents include: (1) protection of drug activity; (2) altered pharmacokinetics, i.e., prolonged serum and tissue half-life; (3) the "depot" effect; and (4) increased endocytosis of conjugates by tumor cells. It is interesting that the potency of conjugates in vivo correlates better with net uptake of the agent in vitro. Arnon,[539] Arnon and Sela,[357]

and Linford and Froese[538] have also noticed the discrepancy between the in vitro and in vivo potency, which is best illustrated by immunotoxins. Although most eradicate tumor cells very selectively and effectively in vitro,[14,340] only in a few studies were they effective in vivo.[29,545,546] Interestingly, these were not "solid" tumors.

VIII. PROSPECTS FOR THE FUTURE

Various agents, from radioisotopes to proteins, have been conjugated to antibodies against widely differing antigens and the conjugates tested for cytotoxicity using experimental systems ranging from simple cell cultures to models in which human tumors have been xenografted in nude mice and occasionally in patients with advanced cancer. Results in vivo have varied from marginally significant inhibition of tumor growth, to prolongation of survival, to cure of a proportion of the treated animals.[14,29,310,469] One may ask: how close are investigators to effective clinical treatment modalities using targeting by antibodies? In this respect, the diagnostic application of antibody localization is substantially ahead of any treatment modality.[547] For many years, the only clinical trial of drug-linked antibodies in the literature was that reported by Ghose et al.[276] The therapeutic application of radiolabeled antibodies in patients was also investigated by only two groups.[470,548] This shows that the concept of antibody-mediated drug targeting is straightforward, but its realization has turned out to be difficult. However, the availability of MAB and the recent success in the use of antibody-linked toxins to eliminate tumor cells from bone marrow have given renewed impetus to investigation. This discussion of the limitations of cancer treatment with antibody-targeted agents and the possible approaches for overcoming some of them should make it clear that in spite of the recent surge of activity in this field there has to be substantial progress in all the components of this approach to cancer therapy before its application to patients. For example, it will be useful to study the TAA profile of various human tumors and document the biological role of the TAA and their behavior in regard to modulation, capping, and endocytosis. An accessible and updated data base on available anti-TAA antibodies and their target TAA will be of help.

The species of origin of a MAB, its class, and subclass may determine effectiveness as a carrier and its immunogenicity and should therefore be documented. It is possible that with time there will be increasing numbers of anti-TAA MAB that have high affinity and adequate tumor specificity. Absolute tumor specificity may neither be achievable nor necessary. It is possible now to isolate the genes that encode for a given MAB and introduce them back into lymphoid or mouse myeloma cells.[549-551] Using plasmids containing Ig cDNA, coexpression of the light and heavy chains of human Ig has been observed in *E. coli*,[552] and the synthesis, processing, and secretion of functional antibodies have been demonstrated with the yeast, *Saccharomyces cervisiae*.[553] In addition to the coding sequences for Ig chains, the mammalian transcription units that have been transfected include genes that allow bacterial selection (e.g., neomycin-resistant gene) and viral or mammalian promoter, splice, and polyadenylation sites.[554] Furthermore, oncogenic DNA viruses (e.g., SV 40 and Epstein Barr virus[555] and defective retroviruses[556,557]) have been used successfully to insert genes into human B-lymphocytes and bone marrow cells. It should be stated that at present the yield of functionally active Ig from these expression systems is very poor for reasons that include the lack of appropriate association between light and heavy chains and adequate post-translational glycosylation and secretion.

DNA recombinant technology has also made possible the construction of chimeric antibody molecules that contain mouse or rat variable regions having a defined specificity and high affinity and human constant regions that could reduce Fc-related antigenicity and confer therapeutically useful functions such as binding to C1Q or Fc re-

ceptors on certain cell types.[558-561] Furthermore, chain switch in Ig can be achieved using a number of methods[562,563] including UV irradiation.[564] This is especially important in the context of MAB of human origin, most of which appear to be of the IgM class.[565] Methods are also becoming available for the production of myeloma transfectants that secrete $F(ab')_2$ or monovalent Ig fragments either completely lacking the Fc moiety[566] or in which the CH_2 and CH_3 domains are substituted by an enzyme moiety.[567] Recombinant DNA technology is thus likely to complement the current hybridoma or B-cell immortalization methodology for the production of anti-TAA MAB for targeting. For example, recombinant technology and methods of site-directed mutagenesis can be used to improve the affinity and other therapeutically desirable qualities of human MAB[563] that have been obtained initially with the use of either lymphocytes that have been antigen-primed in vitro[568] or in vivo (e.g., lymphocytes in tumors or in draining lymph nodes). Genetic engineering is also likely to provide in the not far distant future tailored antibody molecules with appropriate polypeptide chains for optimal conjugation to a given protein toxin or a chemotherapeutic agent. This will include hybrid antibody molecules (already constructed by chemical methods[370]) that have one arm of the IgG directed against the TAA and the other against the protein toxin. It may also be possible to obtain homogeneous populations of molecules that contain the functionally active antigen-binding site of the Ig optimally linked to the toxophore group of a protein toxin or biological products with antitumor activity, e.g., tumor necrosis factor, the gene for which has already been cloned and expression observed in *E. coli*.[569] In contrast to conventionally produced MAB that are products of malignant cells, products of transfectomas are more likely to be free of contaminating oncogenic viruses and nucleic acids.

A number of approaches are possible for production of more effective conjugates and for amplification of their antitumor effects. For example, one can select active analogs of chemotherapeutic agents that have a high influx K_M, e.g., the gamma asparatate derivative of MTX which has a Km for influx into L1210 cells exceeding 300 μM compared to 3.3 μM for MTX.[570] This will minimize uptake of the agent by nontarget cells, as in the case of immunotoxins that contain only A chains. The choice of cytotoxic agent will also depend upon the nature of the TAA, the antibody, the susceptibility of the tumor cells, tumor vascularity, and tumor cell heterogeneity. For anti-TAA antibodies and their fragments such as F(ab) that are not endocytosed, surface active agents and radionuclides should be the agent of choice. For tumors that are relatively avascular or contain antigen-negative tumor cell populations, radionuclides are likely to be more effective than other agents since their tumoricidal effect can extend to neighboring cells.

The choice of the method of conjugation is based presently on the preservation of agent and antibody activities. Better insight into the mechanism of action of conjugated agents, their pharmacokinetics, and their catabolism should allow selection of bonds that are optimal for the effectiveness of the conjugate. Methods need to be devised to prevent the premature dissociation of the carrier antibody and the toxophore and facilitate direct transit of the toxophore to its target molecules in vivo. Regiospecific and pH-sensitive linkages are becoming available and may be of help.[571]

Although the use of a "cocktail" of antibodies has not been very impressive in increasing tumor localization of antibodies,[572] there is evidence that mixtures of ricin-containing immunotoxins against three different T-cell antigens were more effective in inhibiting T-cells in vitro than any single immunotoxin.[509] Furthermore, there may be an increase in the selectivity and the extent of damage with the use of bacterial protein toxins that act cooperatively on mammalian cell membranes.[573] Another approach for amplifying the effect of antibody conjugates is illustrated by the synergistic action of

Pseudomonas exotoxin antibody conjugates and adenovirus[460,574] or ricin-containing immunotoxins against neoplastic T-cells and in vitro active congeners of cyclophosphamide[575] and other cancer chemotherapeutic agents.[575-578] The use of lysosomotropic agents and ionophores that potentiate the effects of conjugates has been discussed.[373,453,460,462,578] Furthermore, the use of biological response modifiers may induce the expression of TAA[173] and vasodilators may improve tumor perfusion.[29]

Another approach will be combination immunochemotherapy. For many cancers, multiple agents are more effective than a single agent. One may render antibody-linked agents more effective by combining more than one free or conjugated drug (including radionuclides). To illustrate, let drugs be designated by A or B and antibodies by X or Y, such that conjugates are A-X, B-Y, A-X-B, etc. One approach is to increase the intracellular concentration of a given drug by exposure to both free and antibody-linked agents so that the drug is internalized by receptors for the antibody and for the drug, e.g., A plus A-X. A variation would be to use A plus B-X, which would show whether the best inhibition is obtained by an increased concentration of a given drug or by two different drugs having different modes of action, e.g., chlorambucil and MTX. Another approach would use two or more conjugates made with one agent by linking to antibody directed against different epitopes that cap and endocytose independently, e.g., A-X plus A-Y. A third approach is a form of combination therapy directed by antibodies. One can link two or more different drugs to a given antibody molecule, e.g., A-X-B, or use a mixture of two conjugates in which different drugs have been linked to separate batches of the same antibody, e.g., A-X plus B-X. Limitations arising from the availability of binding sites for X may be overcome if A and B are linked to antibodies against different epitopes, e.g., A-X plus B-Y.

The effectiveness of antibody-linked agents can be further increased by combination with one or more additional modalities of treatment. It has already been pointed out that antibody conjugates are likely to be most effective against tumor microemboli or cells in circulation and therefore it is essential to reduce the tumor burden by surgery and/or radiation. Furthermore, it is possible that exposure to hyperthermia and/or ionizing radiations (either from an external source or appropriate radionuclides linked to the carrier antibody) may have a complementary or synergistic effect. In the authors' laboratory, it has been observed that exposure to MTX and hyperthermia have additive inhibitory effects on several human tumor cell lines in vitro. Interestingly, MTX arrests cells in the S phase in which they are susceptible to hyperthermia. Thus, local or whole body hyperthermia may be a useful adjunct to immunochemotherapy. Further potentiation of the combined effect of immunochemotherapy and hyperthermia may be possible by linkage to the carrier antibody of appropriate metals that, during magnetic induction heating, have much higher energy absorption than body tissues as well as high thermal conduction properties.[579]

The problems, although formidable, are not insurmountable, especially if this modality of treatment is used for the eradication of those tumor cells in circulation or microemboli that escape and therefore limit the effectiveness of other methods of cancer treatment.

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Exhibit 12

United States Patent [19]

Mattes et al.

[11] **Patent Number: 4,666,845**

[45] **Date of Patent: May 19, 1987**

[54] **MONOCLONAL ANTIBODIES TO OVARIAN, CERVICAL AND UTERINE HUMAN CANCERS AND METHOD OF DIAGNOSIS**

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[58] **Field of Search** ..... 436/519, 520, 548, 809, 436/813, 808; 435/68, 172.2, 240, 241, 948; 935/95, 101, 96, 110, 107; 260/112 R; 530/387, 808

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[57] **ABSTRACT**

Mouse monoclonal antibodies to several cell antigens of human ovarian, cervical and endometrial carcinomas have been produced and characterized. The distribution of the antigens was determined by mixed hemagglutination assays on 153 normal and malignant cell cultures of various types, and by immunoperoxidase staining of frozen sections of 27 normal adult and 24 fetal tissues. five monoclonal antibodies representative of five classes of mAb raised to restricted ovarian, cervical and endometrial cells were tested extensively producing mAb reactive with cancer but not normal cells. One such mAb, MF116 was readily detected in the spent culture medium of metabolically radiolabeled cells. These antibodies, reacting with relatively restricted cell surface antigens, are useful in the analysis of epithelial cell differentiation, in cancer diagnosis and therapy and in tissue typing of normal or abnormal cells.

6 Claims, No Drawings

MONOCLONAL ANTIBODIES TO OVARIAN, CERVICAL AND UTERINE HUMAN CANCERS AND METHOD OF DIAGNOSIS

This invention was partially made with funds provided by the National Cancer Institute under grants CA-26184 and CA-08748. Accordingly, the U.S. Government has certain rights in this invention.

This invention relates to a method for the production of monoclonal antibodies (mAbs) to restrictive antigenic human cell components especially in human ovarian and endometrial tissues. Such mAbs have use in cancer diagnosis and therapy, as well as other cell disorders.

BACKGROUND

Conventional antisera, produced by immunizing animals with tumor cells or other antigens, contain a myriad of different antibodies differing in their specificity and properties. In 1975 Köhler and Milstein (Nature, 256:495) introduced a procedure which leads to the production of quantities of antibodies of precise and reproducible specificity. The Köhler-Milstein procedure involves the fusion of spleen cells (from an immunized animal) with an immortal myeloma cell line. By antibody testing of the fused cells (hybridomas), clones of the hybridomas are selected that produce antibody of the desired specificity. Each clone continues to produce only that one antibody, monoclonal antibody (mAb). As hybridoma cells can be cultured indefinitely (or stored frozen in liquid nitrogen), a constant, adequate supply of antibody with uniform characteristics is assured.

Antibodies are proteins that have the ability to combined with and recognize other molecules, known as antigens. Monoclonal antibodies are no different from other antibodies except that they are very uniform in their properties and recognize only one antigen or a portion of an antigen known as a determinant.

In the case of cells, the determinant recognized is an antigen on or in the cell which reacts with the antibody. It is through these cell antigens that a particular antibody recognizes, i.e. reacts with, a particular kind of cell. Thus the cell antigens are markers by which the cell is identified.

These antigenic markers may be used to observe the normal process of cell differentiation and to locate abnormalities within a given cell system. The process of differentiation is accompanied by changes in the cell surface antigenic phenotype, and antigens that distinguish cells belonging to distinct differentiation lineages or distinguish cells at different phases in the same differentiation lineage may be observed if the correct antibody is available.

The preparation of hybridoma cell lines can be successful or not depending on such experimental factors as nature of the innoculant, cell growth conditions, hybridization conditions etc. Thus it is not always possible to predict successful hybridoma preparation of one cell line although success may have been achieved with another cell line. But it is often true that selected mAb may be representative of a class of mAb raised by a particular immunogen. Members of that class share similar characteristics, reacting with the same cell antigen. Thus the invention includes hybridoma cell lines and mAb with like or similar characteristics.

Progress in defining cell surface antigens is of great importance in differentiation and disease as markers for normal and diseased cells, thereby furthering diagnosis and treatment. Thus work on melanocytes was made possible by the recently discovered technique of culturing melanocytes from normal skin (Eisinger, et al., Proc. Nat'l. Acad. Sci. U.S.A., 79 2018 (March 1982). This method provides a renewable source of proliferating cells for the analysis of melanocyte differentiation antigens. Likewise, a large number of cell lines derived from melanomas have now been established and these have facilitated the analysis of melanoma surface antigens. The advent of mAbs has greatly accelerated knowledge about the surface antigens of malignant melanoma, cell markers on both melanomas and melanocytes have been identified. A panel of typing monoclonal antibodies has been selected which recognizes differentiation antigen characteristics at each stage of development in both melanocytes and melanomas. These differentiation antigens may be used to classify melanocytes and melanomas and to group them into characteristic sub-sets. [Dippold et al. *Proc. Nat'l. Acad. Sci. U.S.A.* 77, 6114 (1980) and Houghton, et al, *J. Exp. Med.* 156, 1755 (1982)]. Immunoassay of melanocytes and melanoma cells within sub-sets is thus made possible.

Initial recognition of differentiation antigens came about through analysis of surface antigens of T-cell leukemias of the mouse and the description of the TL, Thy-1, and Lyt series of antigens. (Old, Lloyd J., Cancer Research, 41, 361–375, February 1981) The analysis of these T-cell differentiation antigens was greatly simplified by the availability of normal T cells and B cells of mouse and man. (See U.S. Pat. Nos. 4,361,549-559; 4,364,932-37 and 4,363,799 concerning mAb to Human T-cell antigens).

The existence of human leukemia specific antigens has been suggested by studies using heterologous antibodies developed by immunization with human leukemic cells [Greaves, M. F. et al. Clin. Immunol. and Immunopathol 4:67, (1975); Minowada, J., et al. J. Nat'l. Cancer Insti. 60:1269, (1978); Tanigaki, N., et al. J. Immunol. 123:2906, (1979)] or by using autologous antisera obtained from patients with leukemia [Garret, T. J., et al., Proc. Nat'l. Acad. Sci. U.S.A. 74:4587, (1977); Naito, K., et al., Proc. Nat'l. Acad. Sci. U.S.A., 80: 2341, (1983)]. The common acute lymphoblastic leukemia antigen (CALLA) which is present on leukemia cells from many patients with non-T, non-B, acute lymphoblastic leukemia (N-ALL), some chronic myelocytic leukemias (CML) in blast crisis and a few acute T-lymphoblastic leukemias (T-ALL) was originally described using conventional rabbit heteroantisera [Greaves, M. F. et al. Supra].

By the autologous typing technique [Garret, T. J., et al. Supra; Naito, K., et al. Supra 1983; Old, L. J. Cancer Res. 41:361, (1981)], antibodies uniquely reacting with ALL cells were found in sera obtained from patients with ALL, and seemed to recognize very similar antigens to CALLA (Garret, T. J., et al. Supra; Naito, K., et al. Supra). Another leukemia associated antigen detected by heterologous antisera is the human thymus leukemia (TL)-like antigen, which is present on thymocytes as well as leukemia cells (Tanigaki, N. et al. Supra). This antigen, is therefore, a normal differentiation antigen which is composed of a heavy chain (MW 44,000–49,000) and light chain (MW 12,000–14,000) similar to the class I HLA antigens (Tanigaki, N., et al.

Supra). These investigations have, however, been hampered by the need for vigorous absorptions with normal tissues as well as the relatively small quantity and low titer of the antisera.

In vitro production of monoclonal antibodies by the technique of Köhler and Milstein, Supra has provided a better system for the identification and detection of leukemia specific antigens. A panel of monoclonal antibodies detecting cell surface antigens of human peripheral blood lymphocytes and their precursor cells have been investigated in detail [Reinherz, E. L., et al. Proc. Nat'l. Acad. Sci. U.S.A. 77:1588, (1980)]. While monoclonal antibodies detecting antigens characteristic for different lymphocyte lineages can be used for classification of human lymphocytic leukemia [Schroff, R. W., et al. Blood 59:207, (1982)], such antibodies have only limited therapeutic applications. Monoclonal antibodies detecting human leukemia associated antigens have also been produced. These include several antibodies detecting the human equivalents of the murine TL antigens. One TL-like antigen is recognized by NA134 [McMichael, A. J., et al. Eur. J. Immunol. 9:205, (1979)], OKT6 (Reinherz, E. L., et al. Supra) and Leu 6 (R. Evans, personal communication). A second TL-like antigen is recognized by M241 (Knowles, R. W., et al. Eur. J. Immunol. 12:676, 1982). Monoclonal antibodies with specificities for common acute lymphoblastic leukemia antigens J-5 (Ritz, J., et al. Nature 283:583, 1980), NL-1 and NL-22 (Ueda, R., et al. Proc. Nat'l. Acad. Sci. U.S.A. 79:4386, 1982) have also been produced. Recently, Deng, C-T, et al. Lancet. i:10, 1982) reported a complement fixing monoclonal antibody (CALLA-2) which reacts with most cultured human T-ALL cell lines and also reacts with most fresh T-ALL cells.

Mouse monoclonal antibodies to human tumor cell surface antigens have been produced in many laboratories (Lloyd, K. O. (1983) In: Basic and Clinical Tumor Immunology, Vol. 1 (R. B. Herberman, Ed.), Nijhoff, The Hague (in press)). The intention of these studies often has been to identify tumor-associated antigens that could be useful in tumor therapy or diagnosis. An inherent difficulty in this approach is the diversity of antigens on the cell surface. Although it has been possible to identify some antigens with a very restricted distribution, antibodies to antigens that elicit very weak immune responses may be missed due to their scarcity. These restricted antigens may be quite difficult to identify. Also, immunization with a complex mixture of antigens such as tumor cells may suppress the antibody response to relatively less immunogenic molecules, in a manner resembling antigenic competition (Taussig, M. J. (1973). Curr. Top. Micro. Immuno. 60:125). Thus production of mAb to restricted cell sites is an especially difficult task. The present invention provide cancer diagnosis and therapy and overcome problems heretofor encountered in the prior art with respect to ovarian and endometrial human cell antigens.

A number of ovarian tumor antigens have been detected using xenogeneic polyclonal sera (reviewed in Lloyd, K. O. (1982) Serono Symposium No. 46 (M. I. Colnagki, G. L. Buraggi and M. Ghrone, Eds.) Academic press. N.Y. pp. 205–211) but none are related to the antigens of the invention. Other laboratories have also described monoclonal antibodies to human ovarian carcinoma different from those of the invention. Bhattacharya et al. (Bhattacharya, M., et al. (1982) Cancer Res., 42:1650–1654) produced an antibody to a saline-extracted antigen detected only in mucinous cyst adenocarcinomas of the ovary and in fetal intestine. Serous cyst adenocarcinomas, the most common ovarian carcinoma, did not contain this antigen. Bast et al. produced an antibody (OC 125) reactive with an antigen present on 6/6 ovarian carcinoma cell lines and one melanoma of 14 non-ovarian cell lines tested. This antibody reacted with sections of 12/20 ovarian carcinomas and was nonreactive with 12 non-ovarian carcinomas and with most normal tissues, including normal adult and fetal ovary. Weak reactivity was observed with adult fallopian tube, endometrium and endocervix (Bast, R. C., et al. (1981) J. Clin. Invest. 68:1331–1336; Kabawat S. E., et al (1983) Amer. J. Clin. Pathol., 79:98–104).

SUMMARY

Monoclonal antibody representative of five separate classes of mAb to ovarian and uterine cancers are described. The antigenic profile of each of these mAbs is presented with both serological and tissue reactivity studies in cancer and normal cell lines and tissue sections. These mAbs form a panel useful for the diagnosis and therapy of cancers of the ovarian and uterine system.

DESCRIPTION

The techniques described below result in the isolation of mAb of several classes; representative mAbs from each of these classes are described and characterized. These techniques can be used to isolate other mAbs from these classes. Thus substantially similar or functionally equivalent monoclonal antibodies having substantially the same characteristics and properties can be produced in accordance with the procedures of the invention. The mAb examples described herein are for illustrative purposes only and are not meant to limit the invention in any way.

Target cells

Cell lines used are listed in Table I. Preparation of cultures of normal human fibroblasts, kidney epithelial cells and melanocytes have been described (Carey, T. E., et al. (1976) Proc. Nat'l. Acad. Sci., U.S.A., 73:3278–3282; Ueda, R., et al. (1979). J. Exp. Med., 150:564–579; Eisinger, M., et al. (1982) Proc. Nat'l. Acad. Sci., U.S.A., 79:2018–2022). Adherent cells were maintained in Eagle's minimum essential medium (GIBCO, Grand Island, NY) supplemented with 2.5% fetal calf serum, 5% newborn calf serum, 100 U/ml penicillin and 1 mg/ml streptomycin. Nonadherent cells were cultured in RPMI 1640 medium supplemented similarly except with 7.5% fetal calf serum. Cultures were regularly tested for mycoplasma and contaminated cultures discarded.

Normal blood mononuclear cells were obtained by centrifuging heparinized blood onto a layer of Ficoll-Paque (Pharmacia, Piscataway, NJ). Total blood leukocytes were obtained by collecting the buffy coat after centrifugation for 10 min at 600 g in 100 microliter capillary tubes.

The origins of cells and tissues is as follows: Drs. Charles Welander, Sloan-Kettering Institute, New York, N.Y. (SK-OV-6 and SK-UT-1), Jorgen Fogh, Sloan-Kettering Institute, New York, N.Y. (SK-OV-3 and SW626), G. Roos, University of Umea at Umea, Sweden (A7 and A10), George Moore, Denver General Hospital, Denver, Colo. (COLO 316) and R. S. Freedman University of Texas, at Houston (2774), Dr. Vir-

ginia Pierce for clinical specimens, the Human Cancer Serology group for cell lines and tissue specimens.

Production of Mouse Monoclonal Antibodies

BALB/c or (BALB/c×C57BL/6)F_1 mice were immunized with the ovarian carcinoma cell lines SK-OV-3, SW626 or 2774, or the endometrial carcinoma cell line SK-UT-1. Intraperitoneal injections of approximately 100 microliters of packed cells were given 2–5 times at intervals of two weeks. Three days after the last injection, the fusion of immune spleen cells with mouse myeloma MOPC-21 NS/1 cells was performed as described (Dippold, W. G., (1980) Proc. Nat'l. Acad. Sci., U.S.A., 77:6114–6118). Initially, cells were plated in 480 wells (Costar 3524, 24 well plates) Hybridoma cultures were subcloned at least two times by limiting dilution in 96 well plates on a feeder layer of normal mouse spleen cells. Culture supernatants were monitored for antibody activity by the anti-mouse Ig MHA (mixed hemagglutination assay) method on a panel of cultured cells consisting of the immunizing cell line and other types of human tumor cells. Cloned hybridoma cells were injected subcutaneously into nu/nu mice. Sera from mice with progressively growing tumors were collected and used for serological and biochemical characterization. Antibody subclass was determined by double diffusion in agar with anti-Ig heavy chain-specific reagents (Bionetics, Kensington, MD).

Serological Procedures

For adherent target cells, 200–500 trypsinized cells were plated in 10 microliters in wells of Terasaki plates (Falcon microtest plates 3034) and allowed to adhere overnight. Nonadherent target cells were attached to the wells by pretreating the wells with concanavalin A (con A, grade IV, Sigma Chemicals, St. Louis, MO) (Mattes, M. J., et al. (1983) J. Immunol. Metho., 61:145–150). The mixed hemagglutination (MHA) assay, using rabbit anti-mouse Ig, has been described (Ueda, R., et al. (1979) J. Exp. Med., 150:564–579). The $CrCl_3$ conjugation procedure has been described (Koo, G. C., et al. (1978) J. Immunol. Meth., 23:197–201), except that undiluted rabbit anti-mouse IgG (DAKO, Accurate Chemicals, Westbury, NY) or the IgG fraction of goat anti-mouse IgM (Cappel Laboratories, Cochranville, PA), at 4.0 mg/ml, was used instead of Protein A. Monoclonal sera were titrated starting at 10^{-3}. To confirm the specificity of antibodies, absorption tests were performed with the immunizing cell line and three melanomas (SK-MEL-28, SK-MEL-37 and MeWo), three astrocytomas (SK-MG-1, SK-MG-3 and U373 MG), three carcinomas (SK-BR-3, SK-LC-6 and Scaber), one T cell leukemia (MOLT-4), one B cell leukemia (Raji) and human erythrocytes. Absorption procedures have been described (Carey, T. E., et al. (1976) Supra.

To test heat stability of antigens, cells were heated 5 min. at 100° C. before performing absorption tests. To test the hydrophobic nature of antigens, cell pellets were extracted with 20 volumes of chloroform methanol, 2:1. Solubilized material was dried and resuspended with sonication in Dulbecco's phosphate-buffered saline (GIBCO), 0.5% bovine albumin (fraction V, Sigma Chemicals), to a volume equal to the original packed cell volume. This suspension was assayed for inhibitory activity of the appropriate antibody.

Immunoperoxidase staining of sections employed 5 micrometer cryostat sections. Air-dried sections were fixed for 10 min at room temperature with 2.0% buffered formaldehyde (Farr, A. G., et al. (1981) J. Immunol. Meth., 47:129–144). A triple sandwich was used routinely which consisted of monoclonal antibody (nu/nu mouse serum at 1/500), biotinylated horse anti-mouse Ig, and complexes of avidin and biotinylated horseradish peroxidase (Vectastain reagents, Vector Laboratories, Burlingame, CA), following procedures recommended by the manufacturer. For particular tissues that had excessive background with this procedure, namely the kidney, liver and pancreas, a double sandwich was used which comprised monoclonal sera at 1/200 and peroxidase-conjugated anti-mouse Ig (DAKO P161) at 1/50. To ensure that fixation did not destroy the antigen investigated, each antibody was first tested on sections of tissue culture cells frozen in 10% dimethylsulfoxide at 50% (packed cell volume/-volume). All antibodies tested were positive in this assay, when the immunizing cell line was used as the target.

Immunofluorescent staining of blood leukocytes in suspension was performed as described (Mattes, M. J., et al. (1979) J. Immunol., 123: 2851–2860) using fluorescein-conjugated goat anti-mouse Ig (Cappel Laboratories) at 1/40, and monoclonal sera at 1/50. Lymphocytes and granulocytes were distinguished by morphology.

Immunoprecipitation Procedures

Each antibody was tested for its ability to precipitate an antigen from detergent-solubilized extracts of the immunizing cell after labeling by three methods: metabolic incorporation of [^{3}H] glucosamine (Ogata, S-I, et al. (1981) Proc. Nat'l. Acad. Sci., U.S.A. 78:770–774), metabolic incorporation of [^{35}S]methionine (Dippold, W. G., et al. (1980) Proc. Nat'l. Acad. Sci., U.S.A., 77:6114–6118), or chloramine T ^{125}I labeling of solubilized cell membranes (Cairncross, J. G., et al, (1982) Proc. Nat'l. Acad. Sci., U.S.A., 79:5641–5645). NP40 solubilization of labeled cells and con A-Sepharose fractionation of labeled extracts, used in some experiments, have been described (Dippold, W. G., et al. (1980), Supra; Ogata, S-I, et al, (1981), Supra, Cairncross, J. G., et al. (1982), Supra), as have immunoprecipitation procedures for ^{125}I-labeled samples, using *Staphylococcus aureus* (Cairncross, J. G., et al. Supra (1982)).

Aliquots of 2×10^6 [^{35}S] cpm from unfractionated cell extracts were handled similarly except that preclearing was omitted. For the con A eluate fraction of [^{35}S]-labeled extracts and for [^{3}H]-labeled extracts, aliquots of 2×10^5 cpm and different washing buffers (Lloyd, K. O., et al. (1981) J. Immunol., 126:2408–2413) were used. Precipitated molecules were extracted with 60 microliter 0.01 M Tris HCl pH 7.2, 2.0% $NaDodSO_4$ (sodium dodecylsulfate), 12.0 mg/ml dithiothreitol (DTT), 15% (wt/vol) sucrose, 0.01% pyronin Y by heating 5 min at 100° C., and analyzed by polyacrylamide gel electrophoresis (PAGE) (Dippold, W. G., et al. (1980) Supra; Laemmli, U. K., (1970) Nature 227:680–685), using 9% gels. For 2-dimensional electrophoresis (isoelectric focusing followed by $NaDodSO_4$-electrophoresis), immune precipitates were extracted and handled as described (Ogata, S-I, et al. (1981) Supra; O'Farrell, P. H., et al. in Method in Cell Biology (Prescott, D. M., Eds.) (1977) Academic Press, New York, Vol. 16, pp. 407–420)). For unreduced samples, DTT was omitted and 14.0 mg/ml iodacetamide was added to samples.

Selection of Hybridomas

Monoclonal antibodies (mAb) MD144, MF61, MF116, ME195 and ME46 were obtained after immunization with ovarian carcinoma cell line 2774, and mAbs MH55 and MH94 were obtained after immunization with endometrial carcinoma cell line SK-UT-1. The heavy chain subclasses of the seven antibodies are: MD144, gamma-sub one; MH55, mu; MF61, mu; MF116, gamma-sub two A; ME46, gamma-sub one; ME195, gamma-sub one; MH94, gamma-sub one. These monoclonal antibodies were initially selected for cloning on the basis of reactivity with the immunizing cell line and lack of reactivity with three melanomas and three astrocytomas. These antibodies were produced from a total of 12 fusions from mice immunized with human ovarian and uterine cancer cell lines. A total of 430 of the supernatants contained antibodies reacting with the immunizing line. Twelve cultures were selected for subcloning, and ten hybridomas were propagated successfully. The properties of seven antibodies are discussed in this publication and two other antibodies, which detected more wide-spread antigens, are described elsewhere (Mattes, M. J., et al. *Hybridoma* (1983) 2:523. None of these mAbs reacted with glycoproteins carrying A, B, H, Le^a, Le^b, X, Y or I blood group structures.

TABLE I

	Reactivity of mouse monoclonal antibodies with cultured human cells and cell lines															
CELLS	mAb MD144				mAb MF61				mAb MF116				mAb MH94			
Ovarian carcinomas																
2774, SK-OV-6, SW 626, SK-OV-3	2	0	0	0[1]	2	0	0	0	2	0	0	0	0	2	2	0
SK-OV-4, Colo 316, A7, A10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SW 626	0				0				0				0			
Endometrial carcinoma																
SK-UT-1	0				0				2				2			
Renal carcinoma																
SK-RC-1, -2, -7, -8	0	0	0	0	2	0	0	0	2	2	2	2	0	0	0	0
SK-RC-9, -10, -12, -17	0		0	0	0	2	2	2	2		2	0	0		0	0
SK-RC-35, Caki-2, SK-RC-4, -6	0	0	0	0	2	2	0	0	0	0	0	0	0	0	0	0
SK-RC-16, -28, Caki-1	0	0	0		0	0	0		0	0	0		0	0	0	
Bladder carcinomas																
253J, SCABER, RT4, VM-CUB-1	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0
VM-CUB-2, 5637, 639-V, J82	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
486-P, JCCSUP	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Colon carcinomas																
SK-CO-10, SW403, SW480, SK-CO-1	0	0	0	0	0	0	0	0	0	0	0	0	2	2	1	0
SW620, SW1222, HT-29	0	0	0		0	0	0		0	0	0		0	0	0	
Breast carcinomas																
CAMA, SK-BR-3, -5, BT-20	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0
BT-474, MCF-7, AlAb, ZR-75-1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
DA-MB-361, MDA-MG-231	0	0			0	0			0	0			0	0		
Lung carcinomas																
SK-LC-9, -15, -1, -2	0	0	0	0	0	0	0	0	0	0	0	0	2	2	0	0
SK-LC-3, -4, -5, -6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SK-LC-7, -8, -10, -13	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SK-LC-14, -16, Calu-1, SK-LC-LL	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SL-LC-12	0				0				0				0			
Cervical carcinoma																
ME 180	0				0				0				1			
Pancreatic carcinomas																
CAPAN-1, -2, ASPC-1	0	0	0		0	0	0		0	0	0		0	2	0	
Neuroblastomas																
LA-N-15, SK-N-sSH, -MC, LA-N-Is	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0
SH-EP1, SK-N-BE(2)	0	0			0	0			0	0			0	0		
Melanomas																
SK-MEL-13, -19, -23, -26	0	0	0	0	0	0	0	0		0	0	0	0	0	0	0
SK-MEL-28, -29, -31, -37	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SK-MEL-75, -93-2, -93-3, -127	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SK-MEL-130, -153, MeWo	0	0	0		0	0	0		0	0	0		0	0	0	
Astrocytomas																
SK-MG-1, -2, -3, -5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SK-MG-6, -8, -11, -14	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SK-MG-15, U138MG, U251MG, U373MG	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Other carcinomas																
SK-HEP-1 (heptoma), G cc-SV	0	0			0	0			0	0			0	0		
T-cell lymphomas and leukemias																
MOLT4, CCRF-HSB-2, CCRF-CEM, 45	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Peer, P12/Ichikawa, HPB-ALL	0	0	0		0	0	0		0	0	0		0	0	0	
B-cell lymphomas and leukemias																
SK-LY-16, -18, Daudi, Ball-1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SK-DLH-2, ARA-10, Raji	0	0	0		0	0	0		0	0	0		0	0	0	
Null-cell leukemias																
NALM-1, -16	0	0			0	0			0	0			0	0		
Myeloid leukemias																
HL-60, K562, KG-1	0	0	0		0	0	0		0	0	0		0	0	0	
Myelomas																

TABLE I-continued

CELLS	Reactivity of mouse monoclonal antibodies with cultured human cells and cell lines											
	mAb MD144			mAb MF61			mAb MF116			mAb MH94		
SK-MY-1, LICR-Lon-HMy-2	0	0	0	0	0	0	0	0	0	0	0	0
Monocytic leukemia												
U937	0			0			0			0		
Normal cells												
Fibroblasts (6), melanocytes	0	0		0	0		0	0		0	0	
Kidney epithelia, 1, 2	0	0		0	0		2	1		0	0	
Non-human cells												
Vero, CHO	0	0		0	0		0	0		0	0	

[1]The symbols listed under the antibodies refer to the titer against the cell line in the corresponding position in the left hand side of the Table. The titer of the antibody was defined as the highest dilution producing at least 50% rosetting in the MHA assay. Symbols are: 2 = a range of $1 \times 10^{-3} - 1 \times 10^{-6}$; 1 = positive reaction but with less than 50% rosetting at 10^{-3} dilution of antibody; 0 = no reactivity at antibody dilution of 10^{-3}.

TABLE II

ANTIBODY	Summary of reactivities of monoclonal antibodies ON TISSUE CULTURE CELLS[1]	ON NORMAL ADULT TISSUE SECTIONS[2]	ON NORMAL FETAL TISSUE SECTIONS[3]
MD144	Ovarian carcinomas (1/8)	Negative	N.T.[4]
MH55	Ovarian (3/8) and uterine (1/1) carcinomas	Negative	N.T.
MF61	Ovarian (1/8) and renal (6/16) carcinomas	Uterine epithelial cells and thyroid colloid positive	Uterine epithelial cells positive
MH94	Ovarian (2/8), uterine (1/1), colon (3/7), breast (1/10), lung (2/18) and cervical (1/1) carcinomas	Pancreas, ureter, breast, prostate, cervix, urinary bladder epithelial cells, sweat and sebaceous glands of skin positive	Stomach, intestine, pancreas, ureter, urinary bladder, uterus and cervix epithelial cells positive
MF116	Normal kidney epithelial cells; ovarian (1/8), uterine (1/1), renal (6/16), bladder (1/10), carcinomas and neuroblastomas (1/6).	Negative	Negative

[1]Antibodies were tested on 153 cell lines of various types (Table I). All tests except those listed were negative.
[2]Antibodies were tested on sections of 27 normal adult tissues: lung, heart, liver, spleen, gall bladder, esophagus, stomach, small intestine, colon, pancreas, kidney, ureter, urinary bladder, adrenal, thyroid, breast, prostate, testes, ovary,fallopian tube, uterus, cervix, placenta, skin, brain, lymph nodes and muscle. Tissues not listed were negative.
[3]Antibodies were tested on 24 normal fetal tissues: lung, heart, spleen, thymus, liver, gall bladder, esophagus, stomach, small intestine, colon, pancreas, kidney, ureter, urinary bladder, adrenal, testes, ovary, fallopian tube, uterus, cervix, skin, brain lymph nodes and muscle. Tissues not listed were negative.
[4]N.T.: not tested.

MD144

Ab MD144 reacted with only a single ovarian carcinoma cell line, 2774, with a titer of 10^4 by immune rosetting; all 152 other cell lines tested were negative (Table I). The antigen was not detected in sections of the normal tissues (Table II). Absorption experiments also did not detect the antigen on any cell type except 2774; in this assay, 1–3 microliters of packed cells was required for nearly complete absorption. The antigen was not destroyed by heating at 100° C. for 5 min, and it was present in the chloroform:methanol extract of 2774 cells. In immunoprecipitation experiments using cell extracts labeled with [^{3}H]glucosamine but not with [^{35}S]methionine, counts were precipitated which migrated at the dye front in both 9% and 12.5% acrylamide gels. These properties all strongly suggest that the antigen is a lipid.

MH55

Ab MH55 is an IgM antibody which reacts weakly with 4/8 ovarian carcinomas (2774, SK-OV-6, A10 and A7) and 1/1 uterine carcinoma (SK-UT-1) with a titer of 10^{-3} or lower; all 148 other cell types tested were negative. Varying the temperature of incubation with antibody, the density of the target cells, and the time interval between target cell plating and testing did not improve the titer or the consistency. We therefore have not done absorption experiments. Ab MH55 did not react with any tissue sections examined but it did react with sections of frozen pellets of A10 ovarian carcinoma cells.

MF61

Antibody MF61 reacted with $\frac{1}{8}$ ovarian carcinoma and 6/16 renal carcinoma cell lines (Table I); the other 146 cell types tested were negative. Absorption experiments revealed no additional positive cell types. Blood leukocytes were negative by immunofluorescence. Absorption tests were unusually sensitive, in that 0.1 microliters of packed cells absorbed nearly completely; negative absorption under our standard conditions

therefore indicates at least a 300-fold lower expression of antigen than on the immunizing cell line. MF61 antigen, like the MD144 antigen, was heat-stable and soluble in chloroform:methanol. The chloroform:methanol extracts were as active as intact cells in absorption experiments. Also, antibody MF61 immunoprecipitated counts from [^{3}H]glucosamine-labeled cell extracts that migrated at the dye front in both 9% and 12.5% acrylamide gels (FIG. 1). It is believed that this antigen is a lipid.

In tissue sections, antibody MF61 reacted with two normal tissues: glandular epithelial cells of the adult and fetal uterus and the noncellular follicles of the thyroid. It also reacted with the follicles of a pig thyroid.

MF116

Three antibodies were obtained from two fusions which react with the same antigen, of which the prototype antibody is MF116. The other two antibodies, ME46 and ME195, are IgG_1, while antibody MF116 is IgG_{2a}. Antibody MF116 reacted with $\frac{1}{8}$ ovarian carcinoma, 1/1 endometrial carcinoma, 6/16 renal carcinoma, 1/10 bladder carcinoma and 1/6 neuroblastoma cell lines. It also reacted with 2/2 normal kidney epithelial cell cultures (Table I). The other 141 cell types tested were negative. Absorption experiments revealed no additional positive cells; 10 microliters of packed cells was required for nearly complete absorption; this result is consistent with a low expression of antigen on the cell surface. Blood leukocytes were negative by immunofluorescence. By immunoperoxidase, MF116 was not detected in any normal tissues examined, including normal kidney, ovary and uterus.

MF116 antigen was immunoprecipitated from [^{3}H]glucosamine or [^{35}S]methionine labeled spent medium from ovarian carcinoma 2774. No antigen was detected in solubilized cell extracts labeled with [^{3}H]glucosamine, [^{35}S]methionine or ^{125}I. This antigen is preferentially shed or secreted in the medium, although it must be present on the cell surface since it is detected in rosetting assays. The molecular weight is 105,000, as estimated by PAGE. If the antigen was not reduced, it migrated slightly faster, indicating some intrachain disulfide bonds. The isoelectric point was determined to be less than pH 4.0, since the antigen migrated at or off the acidic end of the isoelectric focusing gel. This antigen bound to concanavalin A-Sepharose and was eluted with methyl-alpha-D-mannoside. The antigen was destroyed by heating at 100° C., as determined in absorption experiments. MF116 was not detected by immunoprecipitation in the spent medium of two other cell lines (SK-UT-1 and SK-RC-1) that were positive by rosetting.

MH94

MH94 antigen was detected on various carcinoma cell lines, being detected on 2/8 ovarian carcinoma, 1/1 endometrial carcinoma, 3/7 colon carcinoma, 1/10 breast carcinoma, 2/18 lung carcinoma, 1/1 cervical carcinoma and 1/3 pancreatic carcinoma cell lines (Table I). All 142 other cell types tested were negative. Absorption experiments did not reveal additional positive cells; 3 microliters packed SK-UT-1 cells gave nearly complete absorption. Blood leukocytes were negative by immunofluorescence. By immunoperoxidase, MH94 was detected in the acinar and duct lining cells of the pancreas, the epithelial cells of the ureter, breast, pancreas, cervix and urinary bladder and the sweat and sebaceous glands of the skin. It was also found in fetal stomach, intestine, pancreas, ureter, urinary bladder, endometrium and endocervix.

The MH94 antigen was not destroyed by heating to 100° C., but was not detected in a chloroform:methanol extract of cells. It was not precipitated under any conditions tested, which included labeling two cell lines with three isotopes.

These studies describe five specific mAbs detecting highly restricted antigens that are of considerable interest for the analysis of ovarian and uterine tumors (Table II). More broadly reactive antibodies derived from the same fusions, recognizing glycoprotein antigens, were described previously (Mattes, M. J., et al. *Hybridoma* (1983) 2:523. These antibodies generally had higher titers than the ones described here, perhaps reflecting the characteristics of the antigens recognized as discussed below.

Both MD144 and MF61 antigens have properties of lipids or hydrophobic proteins. A substantial fraction of very restricted mouse monoclonal antibodies, produced in several laboratories, have recognized glycolipids (Pukel, C. S., et al. (1982) J. Exp. Med., 155:1133–1147; Nudelman, E., et al. (1982) J. Biol. Chem., 257:12752–12756; Magnani, J. L., et al. (1981) Science, 212:55–56), a result which was quite unexpected on the basis of previous work using whole xenoantisera to human tumor cells. Resistance to heating at 100° C., which is one of their characteristics, could be a property of lipids, carbohydrate determinants on glycoproteins or of exceptional protein determinants. MD144 and MF61 antigens are soluble in chloroform methanol, but this does not distinguish between lipids and hydrophobic proteins (Audubert, F., et al. (1979) Biochem. Biophys. Res. Comm., 91:416–426). Likewise, the immunoprecipitation by mAbs MD144 and MF61 of counts running at the dye front in acrylamide gel electrophoresis, after labeling with [^{3}H]glucosamine, is true of glycolipids as well as small glycoproteins, which would have similar properties. Also, we have evidence that some hydrophobic interactions are not completely disrupted in the presence of the detergents used to solubilize cells, so glycolipids might be co-precipitated by antibodies to hydrophobic proteins or to a nonglycosylated lipid.

MD144 antigen which is found only a single ovarian cancer cell line, is unique. This component is believed to be: a rare human allele, or a rarely expressed gene product, or a mutant form of a normal cell component. Although unique antigens have been demonstrated on chemically induced animal tumors (Baldwin, R. W. (1973) Adv. Cancer Res., 18:1–75) and on human tumors (Old, L. J. (1981) Cancer Res., 41:361–375), present data indicate that these antigens are proteins or glycoproteins (DuBois, G. C., et al. (1982) Proc. Nat'l. Acad. Sci., U.S.A. 79:7669–7673; Carey, T. E., et al. (1979) Proc. Nat'l. Acad. Sci., U.S.A. 76:2898–2902; Real, F. X., et al. (1983) Proc. Amer. Assoc. Cancer Res., 24:233) and therefore differ in this respect from MD144. MF61 has an unusual distribution in normal tissue, being present in the noncellular follicles of the thyroid and in uterine glandular epithelial cells. The dominant antigen of the thyroid colloid is thyroglobulin, but antibody MF61 was not reactive with human thyroglobulin. A second colloid antigen has been described (Balfour, B. M., et al. (1961) Brit. J. Exp. Pathol, 42:307–316), but has not been characterized biochemically. On tumor cells, MF61 is very restricted in its

distribution, being detected only one ovarian carcinoma line and six renal carcinoma cell lines.

MH94 was detected on a small fraction of carcinoma cell lines tested, including carcinomas of the ovary, uterus, colon, breast, lung, cervix and pancreas. The fact that this antigen was detected on only 1/10 breast carcinomas and 2/20 lung carcinomas indicates the importance of testing many cell lines of each tumor type in determining the distribution of an antigen. In frozen sections, MH94 was detected in secretory epithelial cells of many normal tissues.

On tissue culture cells, MF116 was found on normal kidney cells as well as on some carcinomas of the ovary, uterus, kidney, bladder and on one neuroblastoma. The most frequent tumor type that was positive was renal carcinoma, for which 6/16 cell lines were positive. MF116 was not detected in sections of any normal tissue. The presence of MF116 on normal kidney cells in tissue culture and its absence from frozen sections of normal kidney cannot presently be explained, but might suggest that antigen expression is increased in rapidly proliferating cells. MF116 is secreted or shed into the medium by at least some tissue culture cells, and, in fact, is more readily detected by immunoprecipitation using spent medium than with solubilized cell extracts, thus serving as a basis for human cancer diagnosis in this system.

Both MF116 and MF61 show patterns of distribution which seem to be related to the embryological origin of the tissues. Thus, these antigens were detected on tumor cell lines of the ovary, uterus, kidney and bladder but not on cell lines from lung, colon, breast and pancreatic tumors. The former tumors are all from mesoderm-derived epithelia whereas the latter are endodermal or ectodermal in origin. The presence of the antigens in frozen sections of fresh tumor specimens of various types is currently being examined with the immunoperoxidase procedure. It is believed that MF116, MF61 and MH94, but not MD144, are expressed on a proportion of ovarian carcinomas.

The five antibodies described were selected from a large number of hybridoma antibodies produced to ovarian and endometrial cancer cell lines. Each mAb described is representative of a mAb class with similar characteristics. One problem in attempting to produce antibodies to restricted antigens of epithelial tumors is a tendency to produce many antibodies to common, strongly antigenic components. Another factor is that only a small fraction of ovarian carcinomas can be grown and maintained in tissue culture. This invention overcomes the problems. Removing strong antigens from a solubilized cell extract, by the use of immunoadsorbents, before immunization is also possible (Mattes, *Hybridoma* 2:523, 1983).

Diagnosis of cancer by the present invention comprises contacting a tissue containing ovarian and/or endometrial and/or cervical cells with the mAbs recognizing such cell antigens, preferably monoclonal antibodies to one or more cell antigens of the ovarian and/or endometrial and/or cervical antigenic system, and observing the immunoserological or immunopathological antigenic reaction between said monoclonal antibody and said antigen. In a preferred embodiment of the invention, the tissue sample or specimen or part thereof to be contacted is ovarian, cervical or endometrial tissue or cells or parts thereof and the antigenic reaction of the contacted tissue is observed by well known techniques such as immunofluorescence, ELISA, radioactive mAb, rosette formation with sheep or human red blood cells linked to Protein A or to anti-immunoglobulin, direct absorption and the like. In the case of shed antigens, body fluids and/or excretions or secretions can be tested in this manner.

In another preferred embodiment of the invention unknown human Cell specimens are analyzed for mAb reaction with each member of the cell panel using cell sorters for flow cytometry. Thus, the number of cells reacting with fluorescent mAb can be counted. The other well-known observation techniques can be employed to count the number of cells expressing the mAb antigen. In another embodiment of the present invention, the tissue to be assayed is first excised and is then either freshly, or after being frozen or embedded in paraffin by methods well-known in the art, contacted with the monoclonal antibodies of the invention. Observation of the reaction is as before.

In another preferred embodiment of the present invention, the tissue to be assayed comprises the intact body of an individual or whole portion thereof. The antibody, tagged with a radioactive or other energy-producing element, is administered to the individual, and the whole body or part thereof is scanned externally for localization of radioactivity at the site of cancerous cervical, endometrial or ovarian cells.

The present invention also makes possible the treatment of ovarian, cervical, or endometrial tumors in a patient wherein the monoclonal antibody recognizing the cell antigen of cancerous ovarian or endometrial cells, preferably the cell differentiation antigen, is administered to the patient in an amount effective to inhibit the growth or proliferation of cancer cells. In a preferred embodiment of this method, the antibody is tagged with a potentially tissue destructive agent which causes destruction of the cancer cells.

Examples of tissue destructive agents comprise chemotoxic agents, chemotherapeutic agents including vaccines, radionuclides, toxins, complement activators clotting activators and the like.

The invention also enables tissue typing using mAb-tissue immune reactions.

The above examples are for illustrative purposes only and are not meant to limit the scope of the invention.

The hybridoma cell lines producing the monoclonal antibodies of the same designation described above (MF116, MH94, MD144, MH55, MF61, ME46 and ME195) are on deposit and available at Memorial Sloan-Kettering Institute for Cancer Research, 1275 York Ave., New York, N.Y. 10021.

The hybridoma cell lines producing the monoclonal antibodies of the same designation as described above MF116, MH94, MD144, MH55 and MF61 have been deposited with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Md. 20852 a recognized depository on Oct. 28, 1983 and have been given ATCC accession numbers of HB8409 for hybridoma cell line MD144 producing mAb MD144, HB8411 for hybridoma cell line MF116 producing mAb MF116, HB8412 for hybridoma cell line producing MH55 mAb MH55, HB8410 for hybridoma cell line MF61 producing mAb MF61 and HB8413 for hybridoma cell line MH94 producing mAb MH94.

The hybridoma cell lines producing the monoclonal antibodies of the same designation described above ME46 and ME195 have been deposited with the ATCC on Nov. 16, 1983 and have been given ATCC accession numbers of HB8431 for hybridoma cell line ME195

producing mAb ME195 and HB8430 for hybridoma cell line ME46 producing mAb ME46.

What is claimed:

1. Panel of monoclonal antibodies derived by immunization with an ovarian or a uterine cell line for the diagnosis of human uterine, cervical or ovarian cancer wherein the panel consists of at least two of the monoclonal antibodies selected from the group consisting of MF 116 (HB 8411), MH 94 (HB 8413), MD 144 (HB 8409), MH 55 (HB 8412), MF 61 (HB 8410), ME 46 (HB 8430) and ME 195 (HB 8431).

2. Method for diagnosis of malignant human ovarian, cervical or uterine cells which comprises contacting a human ovarian, cervical or uterine cell specimen, or shed antigen containing specimen thereof with one or more of the panel of monoclonal antibodies of claim 1 and immunologically detecting malignant ovarian, cervical, or uterine cells reacting with said monoclonal antibodies.

3. Monoclonal antibody panel of claim 1 wherein the immunogen cell lines are selected from the group consisting of ovarian cell lines SK-OV-3, SW 626, 2774 and uterine cell line SK-UT-1.

4. Hybridoma cell line producing any of the monoclonal antibodies of claim 1.

5. Panel of monoclonal antibodies for the diagnosis of human uterine, cervical or ovarian serous from mucinous cancer wherein the panel is selected from at least two of the group consisting of MF 116 (HB 8411), MH 94 (HB 8413), MD 144 (HB 8409), MH 55 (HB 8412), MF 61 (HB 8410), ME 46 (HB 8430) and ME 195 (HB 8431).

6. Kit for the diagnosis of ovarian, uterine or cervical cancer via shed or intact cell antigens comprisng in package form two or more of the monoclonal antibodies selected from the group consisting of MF 116 (HB 8411), MH 94 (HB 8413), MD 144 (HB 8409), MH 55 (HB 8412), MF 61 (HB 8410), ME 46 (HB 8430) and ME 195 (HB 8431).

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RELATED PROCEEDINGS APPENDIX

None